Targeted Inactivation of p27kip1 Is Sufficient for Large and Small Intestinal Tumorigenesis in the Mouse, Which Can Be Augmented by a Western-Style High-Risk Diet

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ABSTRACT

Mice with a targeted inactivation of both alleles of the cyclin-dependent kinase inhibitor p27kip1 developed both small and large intestinal adenomas when fed a control AIN-76A diet. A Western-style diet that is high in fat and phosphate and low in calcium and vitamin D was also able to initiate adenoma formation in wild-type mice. The combination of p27kip1 inactivation and the Western-style diet was additive in terms of tumor incidence, frequency, and size, and in reducing the life span of the mice. The genetic and dietary combination also resulted in development of adenocarcinoma. Tumor formation was linked to a disruption in homeostasis of the intestinal mucosa, involving increased cell proliferation and decreased apoptosis. There was also decreased goblet cell differentiation as assessed by alcian blue staining and expression of the Muc2 gene, especially in mice fed the Western-style diet, although this differentiation lineage was still present as indicated by expression and staining for intestinal trefoil factor. The inactivation of p27kip1 and the consequent disruption of normal colonic cell maturation in the mucosa was associated with modestly elevated c-myc, cdk4, and cyclin D1 expression. These data establish a fundamental role for p27kip1 in maintenance of intestinal cell homeostasis and in suppressing tumor formation. The data also emphasize the critical role that dietary factors can have in both tumor initiation and progression through interaction with pathways that normally maintain intestinal homeostasis.

INTRODUCTION

Intestinal tumorigenesis arises from perturbations in homeostasis of the intestinal mucosa. This is manifest most clearly as increased cell proliferation compartment, which is normally restricted to the lower two-thirds of the crypt architecture (1, 2). Observations regarding the molecular mechanisms of intestinal tumorigenesis are consistent with this cell biology. Cyclin D1 and c-myc, two genes that encode products drive cells through the cell proliferation cycle in the intestinal mucosa, are direct targets of transcriptional regulation by β-catenin-Tcf4 signaling (3–5). This signaling pathway is nearly uniformly up-regulated in colon tumors because of loss of function of the APC tumor suppressor gene, whose encoded protein, in a complex with axin and glycogen synthase kinase 3β, normally targets β-catenin for degradation (6–9). In addition, up-regulation of the pathway in colon tumors because of mutations in β-catenin itself have also been observed (6).

This fundamental role of enhanced cell cycling in intestinal tumor formation implies that negative regulators of the cell cycle, such as p21WAF1/cip1 and p27kip1, would be effective modulators of tumor formation, and indeed, this has been observed. We (10) demonstrated that the targeted inactivation of p21 increased intestinal tumor formation initiated by an inherited Apc mutation in ApcMin+/− mice, and Philipp-Staheli et al. (11) demonstrated that inactivation of p27 increased intestinal tumors in the ApcMin+/− mouse. In each case, the respective cdki was haplo-insufficient for suppression of Apc-initiated tumors.

We also reported that the inactivation of p21 in the mouse was additive with a Western-style stress diet, a diet that mimics major dietary risk factors for colon cancer in Western societies, in augmenting Apc-initiated tumor formation (10). In the present study, we extended this investigation to the cdki p27 for several reasons: first, although p21 and p27 are both cdkis, they do not serve identical functions in regulating cell cycle complexes that regulate cell cycle progression (12, 13); second, p27 has been demonstrated to have significant functions in colonic cell differentiation outside of its role in regulating cell proliferation, with forced increases and decreases in expression linked to stimulation and inhibition, respectively, of differentiation along the absorptive cell lineage (14, 15); third, decreased expression of p27 is associated with human colon tumor progression and poorer prognosis (16–19), which has not been reported for p21; and fourth, it has been found recently that although p21 is uniformly up-regulated in the differentiation of the goblet cell lineage as well as in the differentiation of intestinal cells that exhibit transepithelial cell transport, p27 elevation is much more pronounced in cells of the latter phenotype (A. Velich, personal communication).

Surprisingly, we found that unlike the case for p21, inactivation of p27 in the absence of an inherited Apc mutation was sufficient to cause development of mouse tumors in both the duodenum and large intestine. This intestinal tumorigenesis by inactivation of p27 was also augmented substantially by feeding the mice the Western-style diet, and was associated with increased proliferation and decreased apoptosis, as well as a decrease in recognizable goblet cells, in the intestinal mucosa. Moreover, both the inactivation of p27 and the Western-style diet modestly increased cyclin D1 and c-myc expression, as well as a down-stream target of c-myc, cdk4.

MATERIALS AND METHODS

p27kip1+− mice, reported previously (20), were mated with each other to generate p27kip1+− or p27kip1−− mice. Genotyping was done using a PCR-based assay of mouse tail DNA, as reported previously. At weaning (~3–4 weeks), the littermates of different genotype were randomized to dietary groups and fed ad libitum either AIN-76A control diet or a Western-style diet that is formulated on the basis of nutrient density to mimic major risk factors for colon cancer in the Western-style diet: high in fat and phosphate and low in calcium and vitamin D (21, 22). Diets were from Harlan-Teklad (Madison, WI).

Animals were maintained on diet for 36 weeks or until they exhibited significant weight loss or other signs of extensive tumor formation. Mice were killed by CO2 overdose and cervical dislocation. The gastrointestinal tract (from stomach to rectum) was opened longitudinally, except for the stomach, which was cut along the greater curvature. The dissected organs were inspected under a dissecting microscope, and the number and location of tumors and macroscopic features of mucosal growth were recorded as described previously (10). Tissues were routinely fixed in formalin and embedded in paraffin

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for histopathological analysis and snap-frozen in liquid N2 for isolation of RNA and protein used in quantitative real time RT-PCR and Western blot analysis, respectively. Proliferation, apoptosis, and goblet cell differentiation in the mucosa were assayed by scoring the percent mitotic cells, the percent terminal deoxynucleotidyl transferase-mediated nick end labeled-positive cells, and the percent alcian blue-stained cells, respectively, as previously described in detail (10).

Total RNA was isolated from the frozen tissues using TRIzol reagent (Invitrogen Life Technology, Carlsbad, CA). The quantity of RNA was determined spectrophotometrically. cDNA was synthesized from Dnase-treated total RNA using TaqMan Multiscribe Reverse Transcriptase (Applied Biosystems, Inc., Foster City, CA). Purified RNA (2 μg) was brought to 10 μl with RNase-free H2O. Reverse transcription mixture (40 μl) was added, containing 1× TaqMan reverse transcriptase buffer, 5.5 mm MgCl2, 0.5 mm deoxynucleotide triphosphate mixture, 2.5 μM random hexamers, 0.4 units/μl RNase inhibitor and 1.25 units/μl reverse transcriptase. The thermal cycler was programmed with the following conditions: incubation for 10 min at 25 °C; reverse transcription at 48 °C for 30 min; and reverse transcriptase inactivation for 5 min at 95 °C.

Quantitative PCR analysis was done using the ABI Prism 7900-HT Sequence Detection System (96-well) and monitored in real time. Sixty ng of cDNA were used as template, to which was added SYBR green dye, 3.0 mm MgCl2, 0.5 μM deoxynucleotide triphosphate mixture, 0.025 units/μl AmpliTaq Gold DNA polymerase, 0.01 units/μl AmpErase UNG, 0.1 μM primers, and dH2O to a final volume of 30 μl. The following primers were used for relative quantification of target gene expression: β-actin, forward 5′-acca-cactgtagcctcatctc-3′ and reverse 5′-gcacatcctgtcagaa-gtc-3′; p27+/−, forward 5′-caacatgtagtgcctaca-aggc-3′ and reverse 5′-agggcttagtgcctaaagtctg-3′; p27−/−, forward 5′-gacagtcgaggctcg-3′ and reverse 5′-gctagcacaaccagtcg-3′; cdk4, forward 5′-acctcaagctggctgacttt-3’ and reverse 5′-gccatctcctgctcgaagtc-3′; p27 kip1, forward 5′-gacagtgagcagttgcg-3’ and reverse 5′-aggggcttatgattctgaaagtcg-3′; and c-myc, forward 5′-gacaagaggcggacacacaa-3′ and reverse 5′-gatgtaggactggtgcttc-3′. The primers for assay of Muc2 and Ift were as described previously (23). Experiments were performed in duplicate for each sample. Each PCR run consisted of a five-point calibration curve and a control containing no template. β-Actin was used as an internal control. The thermal cycling conditions were composed of an AmpliTaq Gold activation step at 95 °C for 10 min followed by 40 cycles of denaturing at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. The relative quantification of target gene was acquired by analyzing the generated data using SDS2.0 software (Applied Biosystems, Inc.). The identity of the expected PCR products was confirmed by agarose gel electrophoresis.

Western blot analysis of steady-state levels of specific proteins was done by standard methods, as we have previously described (24), using the following primary antibodies for detection: anti-c-myc (N-262) and anti-cdk4 (H-22; both from Santa Cruz Biotechnology, Santa Cruz, CA); anti-cyclin D1 (AM29; Zymed Labs, South San Francisco, CA); and anti-β-actin (Sigma, St. Louis, MO). Signal was detected by the enhanced chemiluminescence technique (Amersham Life Science, Piscataway, NJ). Immunohistochemical staining for Ift was previously described in detail (23). Briefly, 4-μg formalin-fixed and paraffin-embedded sections were deparaffinized and rehydrated, quenched with 1.5% H2O2, blocked with 10% normal goat serum, and probed with rabbit anti-Iftp polyclonal antibody (kindly provided by Catherine Tomasetto, Strasbourg, France). Detection was with biotinylated antirabbit IgG (Santa Cruz Biotechnology), followed by incubation with avidin-biotin complex method (Vector Labs, Burlingame, CA) and the substrate 3’,5’-diaminobenzidine combined with hematoxylin counterstaining.

RESULTS

Heterozygote mice that harbor a single inactivated p27 allele were bred to generate mice that were either p27+/− or p27−/−, and these mice were then maintained, after weaning, on AIN-76A control diet or a Western-style diet that is formulated on the principal of nutrient density to mimic the intake of major dietary risk factors for colorectal cancer (high fat and phosphate, low calcium, and vitamin D; Refs. 21, 22). Fig. 1 illustrates that the homozygous inactivation of p27 was sufficient to initiate tumor formation in both the duodenum and the large intestine of mice on the control diet. Fig. 1a shows two tumors, both of which are adenomas, in the duodenum of a p27−/− mouse fed...
suggested that the diet can also initiate genetic or epigenetic changes that initiate tumorigenesis. Tumor initiation has been reported recently for a variation of the Western-style diet that also includes a decrease in methyl group donors (26).

The data of Table 1 illustrate that tumor formation in the $p27^{-/-}$ mice was approximately equivalent in the large and the small intestine. The majority of the tumors that develop in both the duodenum and the large intestine were adenomas, with most being tubular adenomas and some progressing to tubulovillous adenomas (Table 1, Fig. 1a). We also detected adenocarcinomas in both the duodenum and the large intestine of the $p27^{-/-}$ mice maintained on the Western-style diet (Table 1, Fig. 1b). Fig. 1b is a large adenocarcinoma in the colon of a $p27^{-/-}$ mouse fed the Western-style stress diet. In this case, the layers of the intestinal wall have been destroyed by infiltration of the carcinoma. Polyps were found in the stomach of the $p27^{-/-}$ mice fed the Western diet (Table 1), but no neoplastic growths were found in the jejunum or ileum of any of the mice (data not shown).

Fig. 3 shows the consequence of this tumor formation on the life span of the mice. The experiment was carried out for 36 weeks after weaning, and in this time, either the inactivation of $p27$ or the Western-style diet were effective in reducing mouse life span ($P = 0.007$ and $P = 0.039$ for the $p27^{-/-}$ mice or the Western-style diet/wild-type mice, respectively, compared with the wild-type mice fed the control diet). The effects of the Western diet combined with $p27$ inactivation on decreasing life span were additive, consistent with our prior report on inactivation of $p21$ combined with the Western-style diet (10).

To gain insight into the mechanisms by which tumors form in these

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**Table 1 Distribution and histological feature of tumors in the gastrointestinal tract**

<table>
<thead>
<tr>
<th>Organ/diet</th>
<th>$p27^{+/+}$ mice</th>
<th>$p27^{-/-}$ mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIN-76A Western</td>
<td>AIN-76A Western</td>
<td></td>
</tr>
<tr>
<td>Large Intestine</td>
<td>0* 14 (13TA, 1TVA)</td>
<td>10 (10TA)</td>
</tr>
<tr>
<td>Duodenum</td>
<td>0 2 (1TA, 1TVA)</td>
<td>13 (11TA, 2TVA)</td>
</tr>
<tr>
<td>Stomach</td>
<td>0 0</td>
<td>0 6 (6 P)</td>
</tr>
</tbody>
</table>

*Total number of tumors in each group.

**Fig. 2. Tumor formation in the intestine.** The incidence, frequency, and size of intestinal tumors in $p27^{+/+}$ or $p27^{-/-}$ mice fed AIN-76A or a Western-style diet for 36 weeks. (**, $P < 0.05$ comparison to $p27^{+/+}$ mice fed the Western-style diet or $p27^{-/-}$ mice fed the AIN-76A diet, respectively. N, number of mice studied in each group. Arrow indicates 0 tumors for $p27^{+/+}$ mice fed the control diet).
mice, we assayed the levels of cell proliferation and apoptosis in both the duodenum and the large intestine. Only the data for the duodenum are shown, but similar results were found for the large intestine. Consistent with a wealth of data that intestinal tumorigenesis is initiated by a disruption of homeostasis in the mucosa, there were alterations in both these parameters and in the resulting ratio between proliferating and apoptotic cells (Fig. 4, a–c). The increase in proliferation was approximately equal for the p27−/− mice or the Western-style diet/wild-type mice when each was compared with the wild-type mice fed control diet (Fig. 4a). Similarly, the decrease in apoptosis was equivalent for the genetic defect and the dietary perturbation individually (Fig. 4b). This is consistent with the genetic defect and the dietary perturbation each initiating approximately the same number of tumors (Fig. 2). Only modest further changes in proliferation or apoptosis were seen when the p27−/− mice were fed the Western-style diet, but a more substantial further increase was seen when the ratio of the two markers was calculated (Fig. 4c). Interestingly, the Western-style diet alone, or the inactivation of p27, whether in combination with the control or Western diet, decreased the number of detectable goblet cells in the mucosa by ~30% (Fig. 4d), an observation also made for inactivation of p21 (10). However, although there was a decrease in the number of alcian blue-stained goblet cells, decreased mRNA for the major colonic mucin peptide, Muc2, was seen only in the intestinal mucosa from mice that were fed the Western-style diet (Fig. 5a). Moreover, this decreased Muc2 mRNA content was not paralleled by decreased expression of the expression of Itf, another marker of goblet cell differentiation (Fig. 5b). Immunohistochemical evaluation of Itf staining confirmed this observation: there was no decrease in the intensity of staining for Itf or position of cells that express Itf in either the p27+/+ or −/− mice fed either diet (colon data shown in Fig. 5c; similar results not shown for the duodenum).

The increase in proliferation in the intestinal mucosa of mice in which p27 was inactivated or mice fed the Western-style diet or both was reflected in alteration of the machinery that drives cells through the cell cycle. First, as expected, targeted inactivation of the p27 gene resulted in no detectable p27 mRNA in the mucosa of the large intestine of p27−/− mice (Fig. 6a; similar data were obtained for the mucosa of the duodenum, data not shown). Second, there were modest increases in c-myc mRNA expression that are likely linked to the increases in cell proliferation in the mucosa (Fig. 6b). This reached statistical significance for the p27−/− mice fed the Western-style diet compared with wild-type mice fed control diet. These increases were also reflected at the protein level, especially in mice of either wild type or −/− genotype maintained on the Western diet (Fig. 7). There were also modest increases in cdk4 mRNA levels that were statistically significant (Fig. 6c), consistent with the report that this gene is a downstream target of c-myc (27). Again, these increases in cdk4 were also seen by Western blot analysis. Finally, at the protein level, cyclin D1 showed paralleled increases, especially in the p27−/− mice fed the Western diet (Fig. 7), which have the greatest tumor development and the poorest survival. Overall, these changes in the cell cycle machinery were modest, although the cyclin D1 changes exceeded 5-fold in the p27−/− mice fed the Western-style diet, reflecting the alterations in cell maturation that are linked in this model to the development of only one to a few tumors over 36 weeks.

**DISCUSSION**

The principal finding we report is that the targeted inactivation of p27 was sufficient to initiate tumor formation in the duodenum and the large intestine of the mouse. This role of p27 inactivation as an initiator of intestinal tumors has not been previously reported, although it has been reported that p27 inactivation is sufficient for development of pituitary tumors (28). We also observed that, similar to other genetic initiators of tumor formation, maintaining the p27−/− mice on a Western-style diet high in fat and phosphate and low in calcium and vitamin D could profoundly increase the number and size
of intestinal tumors. This observation, along with the fact that the Western diet by itself in these wild-type mice can initiate tumor formation, emphasizes the critical role that diet plays in intestinal tumor formation in developed countries.

As detailed in the “Introduction,” the functions of p21 and p27, especially as regards intestinal cell differentiation, are distinct, and there is a clearer link of decreased expression of p27 to human colon tumor progression and poorer outcome (16–19). It is therefore important that inactivation of p27 was able to initiate tumor formation in the mouse intestine in the absence of an Apc mutation but that inactivation of p21 was not sufficient for tumor formation, although it was effective in enhancing Apc-initiated tumors (10, 29). This function of initiation by inactivation of p27 is consistent with the fact that p27/H11002/H11002 mice also develop pituitary hyperplasia and tumors but that p21 mice do not develop tumors in any tissue (30).

A previous study (11) has demonstrated that inactivation of p27 can augment intestinal tumorigenesis initiated by inactivation of Apc or by dimethylhydrazine but did not report intestinal tumor formation by p27 inactivation alone. However, there were several differences between that study and the experiments presented here. First, data were reported only for p27/H11002/H11002 mice that were initiated by either carcinogen treatment or Apc inactivation, and the age of the p27/H11002/H11002 mice when sacrificed in the absence of any initiating event was not clear. The experiments reported here were in mice maintained on diet for 36 weeks after weaning (~38–40 weeks of age). Second, there is a background strain difference between the p27/H11002/H11002 mice used in the prior study and those used here (129S1/sv × C57Bl/6). Third, because of our interest in the interaction of nutritional and genetic factors in tumor development, we feed our control mice a completely defined diet (AIN76A) that is formulated for maximum growth of the animals, whereas the diet used in the prior study was not specified. Therefore, although these and other potential modulators of tumor formation need to be addressed in experiments with a combinatorial design, the important fact is that inactivation of p27 is capable of initiating intestinal tumor formation.

It is tempting to attribute all of the effects of the inactivation of

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Fig. 5. Analysis of goblet cell markers. In a and b, quantitative real-time RT-PCR was used to assay level of Mac2 and Itf mRNA, respectively, in the flat mucosa of the colon of p27/H11001/H11001 or H11002/H11002 mice fed AIN76A or a Western-style diet. c, immunohistochemical analysis of Itf protein expression (brown stain, ×100) in the flat mucosa of the colon.

Fig. 6. Gene expression in the intestinal mucosa. Quantitative real-time RT-PCR was used to assay the relative level of p27, c-myc, and cdk4 mRNA in the flat mucosa of p27/H11001/H11001 or H11002/H11002 mice fed AIN-76A or Western-style diet. (*, P < 0.05 comparison to p27/H11001/H11001 mice fed AIN-76A diet; **, P < 0.05 comparison to p27/H11002/H11002 mice fed Western-style diet by Mann-Whitney test.)
these cdkis to their activity in inhibiting cell proliferation in the normal mucosa, and perhaps in modulating apoptosis and, indeed, expected alterations in these markers of cell maturation are seen in the flat mucosa of both the p21\(^{-/-}\) (10) and p21\(^{-/-}\) mice (Fig. 4, a and b). However, the role of loss of these inhibitors of the cell cycle may be more complex in intestinal tumor formation. As described in the introduction, the role of the Apc gene product in regulating \(\beta\)-catenin-Tcf4 signaling is paramount in the initiation of intestinal tumor formation (9). Therefore, the phenotype of mice that have a targeted inactivation of the Tcf4 gene is highly instructive. Tcf4\(^{-/-}\) mice die soon after birth, essentially because of differentiation of small intestinal stem cells (31). The loss of the stem cell population abrogates repopulation of the epithelial mucosa upon cell loss and hence the animals bleed to death. This therefore points to a fundamental role of the \(\beta\)-catenin-Tcf4 signaling pathway in intestinal cell differentiation, as well as in proliferation. In confirmation of this hypothesis, down-regulation of this signaling pathway accompanied Caco-2 colon carcinoma cell differentiation (32). Furthermore, forced down-regulation of this signaling pathway in Caco-2 cells by introduction of a wild-type Apc allele, overexpression of E-cadherin, or expression of a dominant negative for Tcf4 was effective in elevating the promoter activity of some genes that are markers for differentiation of intestinal epithelial cells (32). More recently, van de Wetering et al. (33) have clearly demonstrated that \(\beta\)-catenin-Tcf4 signaling directly regulates differentiation of intestinal epithelial cells as well as intestinal cell proliferation. Moreover, these investigators showed that both c-myc and p21 are key players in that high levels of c-myc repress p21 expression, favoring proliferation, whereas low levels of c-myc permit levels of p21 to rise, promoting differentiation (33). Interestingly, in the Apc\(^{1638\Delta}\), p21\(^{-/-}\) mice in which inactivation of p21 leads to increased Apc-initiated tumor formation, there was a significant decrease in alcian blue-stained intestinal goblet cells (10). It is important that we demonstrated in this work that inactivation of p27 also decreased alcian blue-stained cells. However, this was not paralleled by loss of cells that stain for Ift, another goblet cell specific marker. Therefore, similar to work we have reported on tumor formation in mice with a targeted inactivation of the Muc2 gene in which there was also loss of mucin staining but not Ift expression (23), it may be the loss of mucin itself, and not complete ablation of the goblet cell lineage, that is linked to tumorigenesis. However, whether loss of p27 plays a direct role in regulating cell differentiation and mucin synthesis or an indirect role through failure of cells to arrest in the cell cycle as they migrate up the crypt is not yet clear.

The alterations in mRNA expression in the mucosa of components that drive cells through the cell cycle was significant and was also reflected in altered protein levels, although the changes were modest. This likely reflects the significant, although relatively small, shifts in cell maturation in the mucosa (proliferation, apoptosis, and differentiation) that characterize elevation of risk for tumor formation. However, it is important to note that there may be a highly significant link between such changes and the probability of the animals developing a single to a few tumors over 36 weeks of age. This is also precisely the situation in human populations in which \(>90\%\) of the individuals who develop colon cancer also develop only a single tumor in six to seven decades of life, during which time there are \(~10^{12}\) cell divisions in the colon, with no major abnormalities in the molecular or cell biology or functioning of the intestinal mucosa over this time span. Thus, the level of perturbation of homeostasis in the intestinal mucosa and the interactions detected between the genetic pathways and dietary factors in tumor development in this system are likely to be highly relevant to the processes that determine risk for colonic cancer in western society.

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