

\textbf{Abstract}

Targeted inactivation of \(p27^{kip1}\) was sufficient for intestinal tumor formation in mice, but this was strictly a function of diet: tumors formed in \(p27^{-/-}\) or \(p27^{-/-}\) mice fed control AIN-76A diet and were increased by a western-style diet but did not develop in mice fed standard chow diet. When crossed with the \(\text{Apc}^{1638N+/-}\) mouse, \(\text{Apc}^{1-/-} \cdot p27^{-/-}\) or \(\text{Apc}^{1-/-} \cdot p27^{-/-}\) mice not only formed twice as many tumors than the sum of the tumors from mutation at either locus alone, but on AIN76A diet also developed intestinal intussusception, a tumor-associated pathology in patients leading to intestinal blockage that has not been reported for intestinal cancer in mouse models. Moreover, the frequency of intussusception was increased when the compound mutant mice were maintained on the western diet, leading to early death. Despite this more aggressive tumor phenotype generated by inactivation of \(p27\) than by inactivation of another cyclin-dependent kinase inhibitor, \(p21^{\text{WAF1/cip1}}\), the nonsteroidal anti-inflammatory drug sulindac was still effective in inhibiting intestinal tumor formation in \(\text{Apc}^{1-/-} \cdot p27^{-/-}\) or \(\text{Apc}^{1-/-} \cdot p27^{-/-}\) mice, which contrasts with the abrogation of the effects of sulindac in \(\text{Apc}^{1-/-} \cdot p21^{-/-}\) or \(\text{Apc}^{1-/-} \cdot p21^{-/-}\) mice, indicating that \(p27\) is not necessary for tumor inhibition by sulindac. Furthermore, tumor inhibition by sulindac was linked to the induction of \(p21\) expression by the drug, regardless of \(p27\) status, leading to suppression of cell proliferation and promotion of cell differentiation and apoptosis in the intestinal mucosa. (Cancer Res 2005; 65(20): 9363-8)

\textbf{Introduction}

\(p27^{kip1}\) and \(p21^{\text{WAF1/cip1}}\) are both cyclin-dependent kinase (cdk) inhibitors (1, 2), but they do not have identical functions in regulating multifactor complexes that regulate cell maturation. For example, \(p27\) has been shown to have a significant role 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 (3). In addition, there is much better evidence in the literature for level of expression of \(p27\) as a marker of prognosis in human colon cancer than there is for \(p21\) (3–9). Therefore, it was important that we found that, unlike targeted inactivation of \(p21\), targeting of \(p27\) was sufficient for tumor formation, not only in the pituitary, as had been reported (10–12), but also for intestinal adenomas and adenocarcinoma (13). This intestinal tumorigenesis by inactivation of \(p27\) was linked to disruption of intestinal cell maturation caused by elevated expression of \(c\text{-myc}\) and \(c\text{-myc}\) target gene, \(c\text{dk}4\) (13).

Although it was also reported that the targeted inactivation of \(p27\) enhanced adenomatosis polyposis coli (\(\text{Apc}\))–initiated tumor formation in compound mutant mice, that report did not address tumor formation by inactivation of \(p27\) in the absence of a \(\text{Apc}\) mutation (14). Here, we show that this is likely because \(p27\)-initiated tumor formation is strictly dependent on the diet fed the animals. Moreover, we show that compound \(\text{Apc}^{1-/-} \cdot p27^{-/-}\) or \(\text{Apc}^{1-/-} \cdot p27^{-/-}\) mutant mice develop intussusception, an unusual, aggressive tumor-associated pathology seen in patients that has not been previously reported in genetically initiated intestinal cancer in mice. Not only are the frequency and size of the tumors substantially increased in the \(\text{Apc} \cdot p27\) compound model by feeding of a western-style, high-risk diet but also the incidence of intussusception, leading to a severe shortening of life span.

Finally, the nonsteroidal anti-inflammatory drug sulindac has a substantial effect in inhibiting \(\text{Apc}\)-initiated tumors in the intestine of either the human (15, 16) or the mouse (17, 18). We show that the inactivation of \(p27\) does not interfere with this tumor inhibition by sulindac, which contrasts markedly with the abrogation of the effects of sulindac on tumor formation in \(\text{Apc}^{1-/-}\) mice that have a targeted inactivation of the gene encoding another cdk inhibitor, \(p21^{\text{WAF1/cip1}}\) (17, 19). This inhibition of tumorigenesis in the \(\text{Apc} \cdot p27\) compound mice was associated with the ability of sulindac to induce \(p21\), which led to reduced proliferation and apoptosis in both the intestinal mucosa and tumors of \(\text{Apc}^{1-/-}\) mice in which one or both \(p27\) alleles had been inactivated. All of these responses were lost in the \(\text{Apc}^{1-/-} \cdot p21^{-/-}\) and \(\text{Apc}^{1-/-} \cdot p27^{-/-}\) mice.

\textbf{Materials and Methods}

The \(\text{Apc}^{1638N+/-}\) and \(p27^{-/-}\) mouse models and methods for genotyping have been reported (13, 20). \(\text{Apc}^{1638N+}\) mice (C57Bl/6 background) were mated with \(p27^{-/-}\) mice (mixture of 129S1/sv × C57Bl/6) to generate \(\text{Apc}^{1-/-} \cdot p27^{-/-}\) offspring (F1). F1 mice were mated to produce desired genotypes: \(\text{Apc}^{1-/-} \cdot p27^{-/-}\), \(\text{Apc}^{1-/-} \cdot p27^{-/-}\), or \(\text{Apc}^{1-/-} \cdot p27^{-/-}\). At weaning (~3–4 weeks), litters of different genotypes were randomized to different dietary groups and fed, \textit{ad libitum}, either AIN-76A control–defined diet, AIN-76A diet supplemented with 0.02% sulindac, or a western-style diet based on AIN-76A that is formulated on the principle of nutrient density to mimic the intake of major risk factors for colon cancer in the diet of populations in developed countries: high in fat and phosphate and low in calcium and vitamin D (21, 22). Diets were from Research Diets (New Brunswick, NJ). The mice with targeted inactivation of \(p27\) without an \(\text{Apc}\) mutation were also maintained on standard chow diet (LabDiet, Somerville, NJ), AIN-76A diet, or western-style diet.

Mice were weighed weekly and maintained on diet for 16 or 36 weeks, or until they exhibited significant weight loss or other signs of extensive tumor formation. Mice were killed by CO2 overdose and rapidly dissected for evaluation of tumors and fixation of tissues, as described previously (13, 17, 23). Total RNA and protein were isolated from the frozen tissues using TRIzol reagent (Invitrogen Life Technology, Carlsbad, CA), as previously described.

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The quantity of RNA and protein were measured spectrophotometrically, as we described previously (13, 19). cDNA was synthesized from DNase-treated total RNA using Taqman Multiscribe Reverse Transcriptase (Applied Biosystems, Inc., Foster City, CA). Quantitative PCR analysis was done using the ABI Prism 7900-HT Sequence Detection System (96 wells). The primers for p21, p27, and β-actin the amplification conditions for the quantitative real-time reverse transcription-PCR, and methods of data analysis have been reported (13, 19).

Western blot analysis of specific proteins was done by standard methods (13, 19) using the following primary antibodies for detection: anti-p21, anti-p27 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (Sigma, St. Louis, MO). Signal was detected by the enhanced chemiluminescence technique (Amersham Life Science, Piscataway, NJ).

Results

Figure 1A shows that in mice that inherit a heterozygous or homozygous inactivation of the p27 gene (p27+/− and p27−/−, respectively), intestinal tumors formed when the animals were fed a control-defined AIN-76A diet for 36 weeks and increased substantially when they were fed a western-style diet for 36 weeks. Incidence of tumors was very low in these countries. These tumors were both adenomas and adenocarcinomas. Interestingly, the incidence of tumors was nearly twice the sum (i.e., 4.1) of the p27−/− mice fed the AIN-76A diet had at least one intestinal tumor (data not shown). In littermates that were Apc−/−, p27−/−, the mean frequency was 2.67 tumors per mouse (Fig. 2B). This was similar to that of Apc1638N−/− mice on a homogeneous Black 6 background (20), thus reducing the probability that unlinked loci from the p27 mice had a significant effect on the Apc-initiated intestinal tumor formation. However, littermates that were Apc−/− and p27−/− exhibited significantly higher tumor frequencies at 36 weeks of 3.35 and 7.43 tumors per mouse (Fig. 2B; P < 0.05 and P < 0.01, respectively). The 7.43 tumors in the compound Apc−/−,p27−/− mice fed the AIN-76A diet was nearly twice the sum (i.e., 4.1) of the ~2.67 tumors that developed in the Apc−/− mice (Fig. 2B; Apc−/−,p27+/− or Apc−/−,p27+/+) mice on AIN-76A diet for 36 weeks plus the 1.5 intestinal tumors that developed in the p27−/− mice on the same diet at 36 weeks (Fig. 1A; ref. 13). This contrasts with our data on inactivation of another cyclin inhibitor, p21WAF1/cip1, which was only additive with Apc inactivation regarding intestinal tumor formation (23).

Inactivation of p27 also caused more aggressive intestinal tumor development. That is, only 10% of the intestinal tumors in Apc−/− mice were invasive adenocarcinomas, but the incidence was significantly increased to 17% in Apc−/−,p27+/− mice and 19% in Apc−/−,p27−/− mice (P < 0.05), respectively (Table 1).
The western-style diet, which increased tumor formation in the p27−/− and p27+/− mice (Fig. 1A), has been shown to be highly effective in augmenting intestinal tumorigenesis in rodent models (13, 21–24). This was also seen in this study, in that the same western-style diet significantly increased tumor incidence, tumor frequency per mouse, and tumor size, regardless of Apc and/or p27 genotype (Fig. 1A-D). Moreover, the western diet increased the incidence of invasive adenocarcinoma in the p27 wild type, Apc+/−, mice (Table 1). Because the Apc+/− mice with heterozygote or homozygote inactivation of p27 led to early morbidity and death, it was necessary to sacrifice these mice earlier than 36 weeks. Therefore, the increase of adenocarcinoma incidence was likely minimized in the observations on Apc+/−,p27+/− or Apc+/−,p27−/− mice (Table 1). Thus, the western-style, high-risk diet was additive to intestinal tumor formation in the compound Apc,p27 model and in either the Apc or the p27 models alone.

A more detailed examination of the gross pathology of the compound Apc+/−,p27+/− or Apc+/−,p27−/− mice revealed the presence of intussusception of the intestine (Fig. 3). Intussusception is usually caused in humans by the presence of an intestinal tumor (25). Peristaltic seizure and propulsion of the intestine causes the more proximal segment of the intestine, where the tumor is located, to invaginate into the next lower segment. This produces intestinal obstruction. Intussusception was found in 18% (7 of 38) of the Apc+/−,p27−/− mice fed the AIN-76A diet and was significantly increased to 52% (12 of 23) in the Apc+/−,p27+/− mice (Fig. 3D; P < 0.01, compared with Apc+/−,p27−/− mice). No intussusception was found in Apc+/−,p27 wild-type mice fed AIN-76A control diet and was rarely detected in this genotype fed the western-style diet (P < 0.01 and P < 0.001, compared with Apc+/−,p27−/− or Apc+/−,p27+/− mice fed AIN-76A diet, respectively). However, the incidence of intussusception in the Apc+/−,p27+/− or Apc−/−,p27+/− mice fed the western-style diet significantly increased to 29% (8 of 28) and 68% (11 of 16; Fig. 3D; P < 0.05 and P < 0.001, compared with Apc+/−,p27−/− mice fed the western diet, respectively). Most of the intussusception was formed in the jejunum or ileum, and the tumors at sites of intussusception were either adenoma or adenocarcinoma (Fig. 3). In some mice, there were two sets of intussusception. Because of the intussusception, which caused intestinal obstruction, the survival time of the mice dramatically decreased; for example, 50% of the Apc+/−,p27−/− mice fed the western-style diet died at an age of 16 weeks, and none of these mice survived to 30 weeks of age (Fig. 4; P < 0.001).

We have previously studied the role of p21, another cdk inhibitor that is intimately associated with epithelial cell maturation in the intestinal tract, in tumors initiated in Apc+/−, mice, and in the response to sulindac, a nonsteroidal anti-inflammatory drug (17, 23). This was prompted by findings from a number of laboratories, including our own, that p21 was a target of sulindac, for 16 weeks (sulindac), for 16 weeks (B). *, P < 0.05; **, P < 0.01, compared with the mice on AIN-76A diet.

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<th>Table 1. Histopathologic features of the intestinal tumors in Apc−/−,p27+/−, Apc−/−,p27−/−, or Apc−/−,p27+/− mice</th>
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Abbreviations: TA, tubular adenoma; VA, villous adenoma; TVA, tubulovillous adenoma; CA, adenocarcinoma.

*The number of tumors in each group.

**P < 0.05, compared with the mice on AIN-76A diet.

***P < 0.05, compared with Apc−/−,p27−/− mice on AIN-76A diet.

Figure 2. The number of intestinal tumors in the Apc+/−,p27+/−, Apc+/−,p27−/−, or Apc−/−,p27+/− mice fed AIN-76A diet or sulindac supplemented diet (sulindac), for 16 weeks (A) and 36 weeks (B). *, P < 0.05; **, P < 0.01, compared with Apc−/−,p27−/− mice. *P < 0.05, **P < 0.01, compared with the mice fed AIN-76A diet.
inactivation of even one p21 allele was sufficient to eliminate the tumor inhibitory effect of sulindac (17). Moreover, sulindac significantly inhibited tumor progression to malignancy: no adenocarcinomas were seen in Apc+/-,p27++/ or Apc+/-,p27++/ mice fed the sulindac diet (P < 0.05, compared with the mice fed AIN-76A diet), and adenocarcinoma formation was decreased in Apc+/-,p27+/ mice from 19% (17 of 89) to only 6% (2 of 35; P < 0.05) in mice fed the sulindac diet (Table 1). In addition, no intussusception was observed in the mice fed the sulindac-supplemented diet (not shown). Consequently, Apc+/-,p27+/ mice fed AIN-76A diet supplemented with the drug had a life span equivalent to that of Apc+/-,p27 wild-type mice fed just the AIN-76A (data not shown).

In the Apc,p21 model, we have recently shown that there is no induction of p21 even in the Apc+/-,p27++/ mice (17), apparently due to the methylation and functional inactivation of the wild-type p21 allele (19). This strongly suggests that induction of p21 is a critical event in the inhibition of tumors by sulindac. Because tumors were inhibited by sulindac in the Apc+/-,p27++,A pc+/-,p27++, or Apc+/-,p27++/ mice, p21 and p27 mRNA and protein were measured in these animals by quantitative real-time RT-PCR and by Western blot, respectively. First, as expected, p27 mRNA and protein were significantly expressed in the Apc+/-,p27++/ mice, were decreased by 50% in the Apc+/-,p27++/ mice, and were not detected in the Apc+/-,p27++/ mice (Fig. 5A-B). These levels of p27 were not changed by sulindac (Fig. 5A-B), suggesting that p27 is not a target of this drug. Second, p21 was expressed in each genotype,
and at a similar level regardless of p27 genotype, when the mice were fed the control AIN-76A diet (Fig. 5C-D). Thus, there was no compensatory overexpression of p21 in the heterozygous or homozygous p27 mice. However, unlike p27, both mRNA and protein expression of p21 were significantly induced by sulindac regardless of the status of p27 (Fig. 5C-D). Therefore, inhibition of tumorigenesis by sulindac in the Apc−/−,p27+/−, Apc−/−,p27−/−, or Apc−/−,p27+/− mice was linked to the induction of p21. This again provides evidence that a wild-type, functional p27 gene is required for intestinal cells to respond to sulindac to inhibit tumor formation (17, 19), but that they do not require p27 for this tumor inhibition.

**Discussion**

In this report, we have established that the ability of p27 inactivation to cause tumor formation in the intestine is strictly linked to the diet that the mice are fed. Almost no tumors form in p27−/− or p27−/− mice maintained on standard chow diet, but those fed a defined AIN-76A diet show a significant tumor incidence. Both the control AIN-76A diet and the chow diet have been widely used for maintaining rodent growth, but the components differ significantly. The fat in the AIN-76A diet is from corn oil, but the fat in standard chow diet is from soybean oil. The minerals and vitamins in the diets also differ. Both type of dietary fat, as well as other phytochemicals in the chow diet, but not in AIN-76A, have been shown to have profound effects on tumor formation in human populations and in a number of model systems (28–33). Our findings show that dietary factors were critical and additive with inactivation of p27 in initiating intestinal tumor formation. Moreover, we only detected intestinal intussusception in the Apc−/−,p27−/− compound mouse model when the animals were fed the AIN76A control diet, not the chow diet, and the incidence of this pathology was substantially increased by the western-style diet. Intestinal intussusception is seen in intestinal cancer patients but has not been reported for any other mouse genetic model of intestinal cancer.

The western-style diet is formulated on the principle of nutrient density to mimic the intake of major, established risk factors for intestinal cancer in the human (increased fat and phosphate decreased calcium and vitamin D; refs. 21, 22). It is important that both the type of control diet and the western-style diet had such a pronounced effect on tumor formation and pathology initiated by genetic factors, because there is overwhelming evidence in human populations that diet can significantly affect risk for tumor development in different genetic populations (34–36). Therefore, not only is it imperative that diet be considered in the explanation of the large variations in risk for tumor formation at different organ sites in developed and undeveloped countries, but as a practical matter, our data show that use of diets that reflect the human diet of different populations is fundamental to the successful development of mouse genetic models that efficiently and accurately reflect tumorigenesis in the human.

The at-least-additive effects of Apc mutation, p27 mutation, and a western diet on tumor formation in the mouse intestine argues that each affects different steps and/or stages in tumor development. There is increasing evidence that the Apc-initiated transformation is due to the inability of mutant or absent APC protein to successfully target β-catenin for degradation thus resulting in elevated β-catenin-Tcf transcriptional activity (37). Moreover, this pathway seems fundamental not only in controlling epithelial cell growth but also in regulating lineage-specific cell differentiation in the intestinal mucosa (38–43). Consistent with this, inactivation of Mac2, the gene encoding the major gastrointestinal mucin synthesized and secreted by goblet cells, causes tumor formation (44). It is therefore important that the inactivation of either p21 or...
p27 in the Apc−/− mice caused a decrease in mucin-secreting goblet cells (13, 23) and that introducing inactivated p21 into the Muc2−/− mice exacerbated affects on this lineage, causing decreased expression of ITF, another marker of this cell type (45). Thus, although the mechanisms of interaction between different genetic factors and nutritional factors in increasing risk for tumor formation and progression are not yet entirely clear, we predict that these will converge on effects on intestinal maturation and specific aspects of lineage specific differentiation.

Finally, we had been shown that targeted inactivation of p21 aborted the ability of sulindac to inhibit Apc-initiated tumor formation (17), we tested this in the Apc−/− or p27−/− mice model. It was surprising that although inactivation of p27 produced a much more aggressive tumor phenotype than did inactivation of p21, sulindac was still able to inhibit tumor initiation and development of intussusception in the Apc−/− or Apc−/− p27−/− mice as it did in the Apc−/− mice wild type for p27. Thus, p27 apparently plays no role in intestinal cell response to this powerful tumor inhibitor. However, consistent with the critical role of p21 in the response to sulindac (17, 19), p21 mRNA and protein were both induced in the Apc−/− p27−/− compound mouse model regardless of p27 genotype. These data have important implications for predicting clinical response to this drug.

Acknowledgments

Received 6/16/2005; revised 8/8/2005; accepted 8/16/2005.

Grant support: National Cancer Institute grants CA12081, U54 CA100926, CA96605, CA100926 and PO1 13330.

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We thank Drs. J. Pollard (Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY) and A. Koff (Cell Cycle Regulation Laboratory, Memorial Sloan-Kettering Cancer Center, New York, NY) for providing the p27−/− mice.

References

p27kip1 in Intestinal Tumorigenesis and Chemoprevention in the Mouse

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