Localization and Expression of p27KiPl in Multistage Colorectal Carcinogenesis

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ABSTRACT

The cyclin-dependent kinase inhibitor p27KiPl can inhibit the G1 to S transition of the cell cycle and is a putative tumor suppressor. However, our laboratory found that a variety of human cancer cell lines express relatively high levels of this protein and that this is often associated with increased expression of cyclin D1 or cyclin E. Therefore, in the present study we analyzed by immunohistochemistry the expression of p27KiPl in a series of human tissue samples representing various stages of colon carcinogenesis, using 20 samples of normal colon mucosa, 20 hyperplastic polyps, 19 samples of adenomatous polyps, and 40 samples of various types of colorectal carcinomas. Parallel immunostaining was done for cyclin D1 and also for Ki67 to evaluate cell proliferation. An additional 17 human colon carcinoma samples, together with paired adjacent normal mucosa samples, were analyzed for levels of expression of the p27KiPl protein by Western blot analysis, and 7 of these pairs of samples were examined by Northern blot analysis for levels of p27KiPl mRNA. We did not find a positive or negative correlation between p27KiPl expression and cell proliferation in the normal mucosa and tumor samples. There was, however, an inverse correlation between p27KiPl and Ki67 expression in the lymphoid follicles present in the colonic mucosa. There was no evidence for a consistent increase or decrease in p27KiPl expression in the mucosal cells during colon carcinogenesis, because the mean values for percentage p27KiPl-positive cells were similar in the normal mucosa, adenomatous polyps, and carcinoma samples. This is in contrast to Ki67 and cyclin D1 expression, which did show significant increases in mean values with tumor development. A subset (35%) of the carcinomas displayed diffuse cytoplasmic staining, in addition to nuclear staining, for p27KiPl, and in these cases the percentage of cells that were positive for p27KiPl was higher than in cases that had only nuclear staining. There was a significant correlation between p27KiPl expression and tumor grade; i.e., well and moderately differentiated carcinomas had high p27KiPl expression, whereas poorly differentiated carcinomas had lower expression. The Western blot analysis data on p27KiPl expression confirmed this correlation. Comparisons of Northern and Western blots did not show a correlation between the level of p27KiPl mRNA and the corresponding protein, a finding consistent with evidence that the p27KiPl protein is regulated mainly via a posttranscriptional mechanism. The immunostaining studies revealed a significant correlation between high p27KiPl protein expression and high cyclin D1 expression in the adenomatous polyps and in the subset of carcinomas that had only nuclear p27KiPl expression. This may reflect the existence of a homeostatic feedback mechanism that is lost in the high-grade carcinomas that express low levels of p27KiPl.

INTRODUCTION

Abnormalities in various components of the cell cycle-regulatory machinery have been found in several types of human cancer. Thus, amplification and/or increased expression of the cyclin D1 gene have been found in esophageal, head and neck, hepatic, breast, and colon cancers; cyclin E is often deregulated in colon, breast, and prostate tumors; and Cdk2 is often overexpressed in sarcomas, gliomas, and colon carcinomas (1–11). There is also increasing evidence for alterations in the expression of specific CKIs in human cancers. Six CKIs, which form two distinct classes, have been identified in mammalian cells. The first class includes the INK4 proteins: p16INK4A (12), p15INK4B (13), p18INK4C (14), and p19INK4D (15). Each of these proteins can form complexes with Cdk4 and Cdk6 (16). Overexpression of INK4 proteins can block cells in G1 (17), presumably through inhibition of cyclin D/Cdk activity. Inactivation of the p15INK4B or p16INK4A proteins results in accelerated entry into S phase and appears to contribute to cellular transformation, suggesting that the INK4 proteins function as tumor suppressor genes (18). Indeed, a high frequency of p15INK4B and p16INK4A gene deletions occur in specific tumors and in tumor-derived cell lines (15, 19, 20). Moreover inactivation of p16INK4A expression due to hypermethylation of this gene has also been seen in human tumors (21), and p16INK4A-deficient mice develop spontaneous tumors at an early stage and are highly sensitive to carcinogens (22).

The second class of CKIs includes p21Cip1 (23, 24) p27KiPl (25, 26), and p57Kip2 (27). The p21Cip1 gene is located on chromosome 6p, and the encoded protein inhibits a variety of cyclin-Cdk complexes, including cyclin D-Cdk4, cyclin E-Cdk2, and cyclin A-Cdk2. Its expression is regulated by the tumor suppressor p53 (28) although it can also be induced by p53-independent mechanisms (29) and may be involved in cellular senescence (30). No mutations in the p21Cip1 gene have been described thus far in human cancers (31), and p21-deficient mice undergo normal development, although fibroblasts from these mice are defective in G1 arrest in response to DNA damage and nucleotide pool depletion (32, 33). Two additional CKIs that are structurally related to p21Cip1 have been identified, p27KiPl and p57Kip2. The latter gene (27) has not been studied in detail in human cancers. The p27KiPl protein associates with cyclin D-Cdk4, cyclin E-Cdk2, and cyclin A-Cdk2 complexes and can inhibit their activities (34). Several studies have demonstrated the importance of this protein in controlling G1 progression during the cell-cycle. Thus, p27KiPl interfered with G1 progression when its level was increased by camp agonists in macrophages (35), by rapamycin in T lymphocytes (36), by IFN-γ in mammary epithelial cell lines (37), or by IFN-β in the TMK-1 human gastric cell line (38). On the other hand, abrogation of p27KiPl function suppresses quiescence in Chinese hamster cell lines (39). This protein appears to play a role in both cell growth and differentiation, because ectopic overexpression of p27KiPl induces differentiation in some cell lines (40). It is of interest that the expression of p27KiPl is regulated mainly at the posttranscriptional level via a ubiquitin-proteasome mediated proteolysis mechanism (41, 42). The p27KiPl gene is located on chromosome 12p (43–45). Although the role of p27KiPl in negative regulation of cell proliferation suggests that it may function as a tumor suppressor gene, several authors have noted the absence of mutations in this gene in a variety of tumors
(43–45). Therefore, this protein may play a positive role in the growth of some tumors.

Indeed, we found that a variety of human carcinoma cell lines express relatively high levels of p27kip1 (46–50). In a series of esophageal cancer cell lines, there was a positive correlation between the level of the cyclin D1 protein, which would be expected to enhance growth, and the level of the p27kip1 protein, which would be expected to inhibit growth (46). Human colon and breast cancer cell lines also expressed high levels of the p27kip1 protein, but this protein was expressed at low levels in three normal mammary epithelial cell lines (47–50). Ectopic overexpression of cyclin D1, or cyclin E, in mammary epithelial cell lines that express low levels of both of these cyclins was associated with increased expression of p27kip1 (47–50). The reciprocal effect was also seen, because when we used an antisense cyclin D1 cDNA to reduce the expression of cyclin D1 in an esophageal or colon cancer cell line, this led to reduced levels of expression of the p27kip1 protein (51, 52). On the basis of these findings, we postulated the existence in some cell types of a feedback inhibitory loop between cyclin D1 or E and p27kip1, the function of which might be to maintain a homeostatic balance between positive and negative regulators of the G1 to S progression of the cell cycle (46–50, 52).

In view of the above findings, the present study used immunohistochemistry to compare the topology and frequency of expression of the p27kip1 protein to that of cyclin D1 and Ki67, a marker of proliferation (53), in a series of samples of normal human colonic mucosa, hyperplastic polyps, adenomatous polyps, and colorectal carcinomas. Western and Northern blot analyses for levels of expression of the p27kip1 protein and mRNA, respectively, were also done on a smaller set of paired colorectal carcinoma and normal mucosa samples. We also evaluated possible correlations between the findings obtained with the carcinomas and various clinical and pathological parameters. Our findings suggest that p27kip1 plays a complex role in colon carcinogenesis, which is not simply related to its inhibitory effects on cell proliferation.

MATERIALS AND METHODS

Patients. A total of 98 cases were investigated. Patients underwent surgery at the Columbia-Presbyterian Medical Center (New York, NY; 88 cases) or at Osaka University Medical School (Osaka, Japan; 10 cases) from February 1995 to May 1996. The mean age was 67.74 (range, 32–92 years). The tissues were removed endoscopically or obtained at the time of surgery and then routinely processed. They were formalin-fixed and paraffin embedded for the immunohistochemical study or immediately frozen at −80°C for the Western and Northern blot analyses. Samples were classified for histological type and Dukes’ stage according to standard criteria. Adenomas and colorectal carcinomas were graded according to the WHO grading system. The samples included normal mucosa (n = 35) from the resection margin of colorectal carcinomas, which would be expected to enhance growth, and the level of the p27kip1 protein, which would be expected to inhibit growth (46). Human colon and breast cancer cell lines also expressed high levels of the p27kip1 protein, but this protein was expressed at low levels in three normal mammary epithelial cell lines (47–50). Ectopic overexpression of cyclin D1, or cyclin E, in mammary epithelial cell lines that express low levels of both of these cyclins was associated with increased expression of p27kip1 (47–50). The reciprocal effect was also seen, because when we used an antisense cyclin D1 cDNA to reduce the expression of cyclin D1 in an esophageal or colon cancer cell line, this led to reduced levels of expression of the p27kip1 protein (51, 52). On the basis of these findings, we postulated the existence in some cell types of a feedback inhibitory loop between cyclin D1 or E and p27kip1, the function of which might be to maintain a homeostatic balance between positive and negative regulators of the G1 to S progression of the cell cycle (46–50, 52).

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epithelial cells that displayed nuclear immunostaining for p27Kip1, with a mean ± SD of 45.7 ± 18.5% (Fig. 1a). The median value was about 50%. Therefore, in our subsequent analyses, samples in which 50% or more of the cells were positive for p27Kip1 staining were defined as "high expressors," and those with less than 50% were defined as as "low expressors." The 50% cutoff value was also used by previous investigators to distinguish high and low p27Kip1 expression, and in their studies, it had prognostic significance for cases of colon cancer (52). A typical histological section of normal colonic mucosa is shown in Fig. 2A. The positive colonic epithelial cells displayed moderately intense nuclear staining and were distributed throughout the length of the crypts. This included the basal proliferative compartment, as well as the middle and upper thirds of the crypts, which contain differentiated cells. On the other hand, nuclear staining for Ki67 was confined to the cells in the lower one-third of
Fig. 2. Representative histological sections demonstrating topology of p27Kip1 and Ki67 immunostaining. A, normal colonic mucosa. p27Kip1 staining is nuclear and is located in cells along the entire length of the crypts, including the basal proliferative region. In the lamina propria, lymphoid cells show very intense staining for p27Kip1, ×250. B, well differentiated colon carcinoma. This tumor displays numerous cells that have nuclear staining for p27Kip1, ×250. C, lymphatic follicle in normal colonic mucosa. Ki67 immunostaining is exclusively present in the germinal center of the follicle. ×250. D, serial section of the lymphatic follicle shown in C. p27Kip1 immunostaining is exclusively present in the peripheral, nonproliferating area. ×250. The immunostaining used the avidin-biotin complex technique and 3,3'-diaminobenzidine development (brown). Cells were lightly counterstained with hematoxylin. For additional details, see "Materials and Methods."

the crypts (data not shown). The mean value for the percentage of normal colonic cells that were positive for Ki67 was 10.2 ± 3.9% (Fig. 1c). The mean Ki67 value for the group of samples that had high p27Kip1 expression was essentially the same as that for the low p27Kip1 group (10.0 ± 3.1% versus 10.9 ± 5.5%). Therefore, by this criterion, as well as by our findings on the regions of the crypts in which p27Kip1 was expressed, there was no correlation between extent of cell proliferation and p27Kip1 positivity.

A striking finding was the intense staining of lymphoid cells for p27Kip1 (Fig. 2A). This was also seen in the polyps and carcinomas. Within the lymphatic follicles of the colonic mucosa, the germinal center showed an abundance of Ki67 staining but no p27Kip1-positive cells, whereas in the peripheral zone, there was an inverse pattern of staining (Fig. 2, C and D). Thus, in contrast to the colonic epithelial cells, in the lymphoid cells, the expression of these two proteins was mutually exclusive, suggesting that proliferating lymphoid cells, but not proliferating colonic epithelial cells, markedly down-regulate the expression of p27Kip1.

**Hyperplastic Polyps.** The 20 hyperplastic polyp samples also displayed a wide range of values for percentage of p27Kip1-positive cells (20–70%), with a mean value of 49.1 ± 17.4%, similar to that found in the series of normal mucosa samples (Fig. 1a). The distribution of p27Kip1-positive cells along the length of the crypt was also similar to that seen in the normal mucosa. Ki67 staining was prominent in the lower one-third, and isolated stained cells were observed in the middle one-third of the crypts (data not shown).

**Adenomatous Polyps.** The 19 adenomas showed a wide range of p27Kip1-positive cells with a mean value of 43.4 ± 19.8% (Fig. 1a). The cells with p27Kip1-positive nuclei were located throughout the lesions, in the superficial epithelium and also along the whole length
of the crypts. Ki67 staining was present in the basal area and extended to the middle and upper thirds of the crypts. Thus, even in the adenomas, p27<sup>Kip1</sup>-positive cells were present in areas of both high and low cell proliferation.

**Colorectal Carcinomas.** The 40 colorectal carcinomas showed a range of nuclear-positive p27<sup>Kip1</sup> cells of 10–80%, with a mean value of 55.8 ± 18.3% (Fig. 1a). The cells with p27<sup>Kip1</sup>-positive nuclei were distributed through the tumor section. The intensity of staining was generally moderate, with only a few positive tumor cells showing stronger immunoreactivity (Fig. 2B). Ki67-positive cells were irregularly scattered throughout pseudoglandular structures. There was not an inverse correlation between regions of high p27<sup>Kip1</sup> and high Ki67 expression among the tumor cells, nor was there a significant difference between Ki67 mean values between the carcinomas that expressed a high or low percentage of p27<sup>Kip1</sup>-positive nuclei (35.4 ± 15.3% versus 26.5% ± 12.4%, respectively).

A subset (35%) of the carcinomas displayed not only nuclear staining for p27<sup>Kip1</sup> but also diffuse cytoplasmic staining. This cytoplasmic staining was peculiar to the carcinomas because it was not seen in the normal mucosa samples, hyperplastic polyps, or adenomatous polyps. Furthermore, among the group of carcinomas that displayed both nuclear and cytoplasmic staining, the percentage of cells that were positive for p27<sup>Kip1</sup> was significantly higher than in the group of carcinomas that had only nuclear staining (71.4 ± 8.3% versus 47.3 ± 16.5%; P < 0.0001; Fig. 1b).

Table 1 summarizes possible correlations between the extent of p27<sup>Kip1</sup> expression among the 40 colorectal carcinomas and various clinical and pathological parameters. As described above (also see "Materials and Methods"), high expressors were defined as tumors in which 50% or more of the cells were positive for p27<sup>Kip1</sup> staining, and low expressors were defined as those in which this value was less than 50%. We found a significant correlation between carcinomas that had high p27<sup>Kip1</sup> levels and the well and moderately differentiated tumor grades (P = 0.0007; Table 1). Furthermore, p27<sup>Kip1</sup>-positive cells were located predominantly in areas of tumors with low-grade architectural and cytological alterations. The mean values for percentage of positive p27<sup>Kip1</sup> tumor cells were 63.0 ± 16.2% in the well and moderately differentiated subsets of tumors and 43.7 ± 14.82% in the poorly differentiated subset (P = 0.0020). A statistically significant association was also seen between p27<sup>Kip1</sup> expression and increased tumor size (P = 0.0292; Table 1), but the validity of this association is uncertain because of the small number of cases in one group of this contingency category. No other significant association between p27<sup>Kip1</sup> expression and the other clinicopathological parameters was observed (Table 1). As mentioned above, some of the carcinomas displayed both nuclear and cytoplasmic p27<sup>Kip1</sup> staining, but the number of these cases was too small to search for possible clinical and pathological correlations unique to this subset. However, we observed that cases with only nuclear staining were more frequently the well and moderately differentiated tumors, as described above, whereas the cases with both nuclear and cytoplasmic staining were equally distributed between the low- and high-grade tumors (data not shown).

**Immunohistochemical Studies on Cyclin D1 Expression.** In view of previous evidence that cyclin D1 is often overexpressed in colorectal carcinomas (7, 8), it was of interest to also evaluate by immunostaining cyclin D1 expression in the above set of samples. The results are summarized in Fig. 1d. The normal mucosa samples displayed less than 20% of cyclin D1-positive cells, with a mean value of 9.7 ± 3.9%. The immunostaining for cyclin D1 was generally weak and was mainly nuclear, but some samples also showed cytoplasmic staining, as described previously (8). Positive cells were mainly located in the lower one-third of the crypts but were also present in other regions, including the luminal surface (data not shown). Hyperplastic polyps, adenomas, and carcinomas displayed moderate or intense staining and a higher percentage of positive cells when compared to the normal mucosa samples. Thus, the mean values for percentage of positive cells in the hyperplastic polyps, adenomatous polyposis, and colorectal carcinomas were 20.3 ± 10.2%, 28.6 ± 18%, and 34.5 ± 16.1%, respectively (Fig. 1d). The differences in the mean values between the normal mucosa and the adenomatous polyps or the colorectal carcinomas were highly significant (P < 0.0001 in both cases). There was also a significant increase in the mean values for Ki67 expression between normal mucosa and adenomatous polyps or normal mucosa and colorectal carcinomas (P = 0.0364 and P < 0.0001, respectively; Fig. 1c). However, this was not true for p27<sup>Kip1</sup> (P = 0.8111 and P = 0.0651, respectively; Fig. 1a). Moreover, an analysis across all categories of tissue samples showed significant increases in the mean values of Ki67 and cyclin D1 during the progressive stages of colon carcinogenesis (P < 0.001 in both cases), but this was not true for p27<sup>Kip1</sup> (P = 0.1227).

Among the colorectal carcinomas, we found no correlation between cyclin D1 expression and the various clinical and pathological parameters listed in Table 1 (data not shown). In the series of adenomatous polyps, there was a significant positive association between cyclin D1 and high p27<sup>Kip1</sup> expression (P = 0.0408) by the χ² test, but no significant association was seen between these two proteins in the series of colorectal carcinomas using either the χ² test (P = 1.000) or the linear regression analysis (r² = 0.007). It is of interest that 8 of the 9 (88%) carcinoma samples that showed high expression of both of these proteins had only nuclear staining for p27<sup>Kip1</sup>, whereas only 1 of these 9 (12%) carcinomas showed nuclear plus cytoplasmic staining for p27<sup>Kip1</sup> (data not shown). Thus, there is a significant association between increased expression of these two proteins in a subset of the carcinoma samples.
Western and Northern Blot Analyses of p27Kip1 Expression. To extend the results obtained by immunohistochemical analysis of histological sections, we also examined by Western blot analysis an additional 17 primary colorectal carcinoma surgical specimens, together with paired adjacent normal colonic mucosa samples obtained from the same cases, for levels of expression of p27Kip1 in total tissue lysates. The results obtained were quantitated by densitometry. As in the immunostaining studies, both the normal colonic mucosa and carcinoma samples displayed a wide range of expression of the p27Kip1 protein but similar mean values (Fig. 3a). Seventeen of the 17 carcinomas (41%) had higher levels of the p27Kip1 protein than their corresponding paired adjacent normal mucosa samples. The remaining carcinoma samples had levels of this protein that were equal to or less than those in the paired normal mucosa sample.

In view of the results obtained with p27Kip1 immunostaining (Fig. 1b), we also analyzed these Western blot data for possible correlations with the histological grade of the carcinomas. Again, we found a correlation between high p27Kip1 protein expression and carcinomas that were well or moderately differentiated (Fig. 3b). Thus, the mean densitometric value for the level of the p27Kip1 protein in the well and moderately differentiated carcinomas was significantly higher than that of the poorly differentiated carcinomas (1711 ± 1078 versus 315 ± 177; P = 0.0324; Fig. 3b). We also performed Northern blot analysis on mRNA extracts prepared from seven of these paired normal mucosa and carcinoma samples. All of the samples revealed a characteristic 2.5-kb p27Kip1 mRNA band. In four (56%) cases, the level of this mRNA was 1.8–15.6-fold higher in the carcinoma sample than in the paired adjacent normal mucosa. A comparison between Western and Northern blot analyses revealed that there was no consistent correlation between the p27Kip1 protein level and mRNA levels in each sample (data not shown).

DISCUSSION

The present study indicates that in normal human colonic mucosa and during colorectal carcinogenesis, p27Kip1 expression does not correlate with cell proliferation. Thus, our immunostaining studies indicated that in normal colonic mucosa, p27Kip1 was expressed in both the basal proliferative region of the crypts and in the differentiated upper regions of the crypts (Fig. 2a), in contrast to Ki67, which was expressed only in the basal region of the crypts. Although a series of hyperplastic polyps, adenomatous polyps, and colorectal carcinoma samples displayed a progressive increase in the mean score for Ki67 expression, there was no significant upward or downward trend in the respective values for p27Kip1 expression (Fig. 1, c and a, respectively). In addition, among the colorectal carcinomas, there was no correlation between p27Kip1 and Ki67 expression. The absence of an inverse correlation between p27Kip1 expression and extent of cell proliferation is somewhat surprising in view of the known role of this protein as a negative regulator of the cell cycle (25). This finding is, however, consistent with our previous evidence that p27Kip1 is often expressed at relatively high levels in exponentially growing human cancer cell lines (46–50). At the same time, we did find that well and moderately differentiated colorectal carcinomas had higher mean values for p27Kip1 than poorly differentiated carcinomas (Table 1). Western blot analyses for levels of expression of the p27Kip1 protein done on 17 colorectal carcinoma samples and paired adjacent normal mucosa samples also indicated no consistent increase or decrease in the tumor versus the normal samples and also confirmed the association of high expression in the low-grade carcinomas (Fig. 3b).

During the course of our studies, other investigators have reported findings on p27Kip1 expression in human colon, breast, and non-small cell lung carcinomas (54–56, 60, 61) and gastric carcinomas4 that are consistent with our results. These studies also found no correlation between the level of p27Kip1 expression in tumors and the extent of cell proliferation, but there was a positive correlation between high p27Kip1 expression and low-grade histology and/or a more favorable prognosis. The previous studies on colon cancer (54, 60) did not examine colonic polyps or paired colorectal carcinoma and adjacent normal mucosa samples. In a subset of our paired samples, we also did Northern blot analyses for p27Kip1 mRNA and found no consistent correlation between the level of p27Kip1 mRNA and protein in the

same sample. This finding is consistent with evidence that cellular levels of this protein are regulated mainly via posttranscriptional mechanisms, including ubiquitin-proteasome-mediated proteolysis (41, 42, 54). Indeed, there is evidence that the low levels of p27Kip1 seen in some colon and lung carcinomas are due to increased activity of this proteolytic mechanism (54, 61).

Thirty-five % of the colorectal carcinomas displayed not only nuclear immunostaining for p27Kip1 but also diffuse cytoplasmic staining. This group of carcinomas also displayed a higher percentage of cells that were positive for nuclear p27Kip1 staining (Fig. 1b). At the present time, it is not clear whether this represents simply leakage of the protein from the nucleus or proteolytic degradation. Alternatively, because there is evidence that p27Kip1 and p21Cip1 direct the accumulation of cyclin D1 to the nucleus (61), the presence of p27Kip1 in the cytoplasm may reflect a defect in nuclear translocation. Other investigators have also noted cytoplasmic immunostaining for p27Kip1 in some tumor samples (54). In vitro studies in colon cancer cell lines may clarify the significance of this finding.

In this study, we did not examine the level of expression of another CKI p21Cip1, but other investigators have studied the expression of this protein in normal human colonic mucosa and during colorectal carcinogenesis. In the normal colonic mucosa, p21Cip1 protein (62, 63) and mRNA (64) are expressed mainly in the upper one-third of the crypts and the superficial differentiated cells and not in the Ki67-positive basal proliferative compartment. This is in contrast to our findings in the present study, in which p27Kip1 expression did not show a reciprocal relationship with Ki67 expression in normal colonic mucosa. In the previous studies on colorectal carcinomas, p21Cip1 expression was heterogeneous. High expression was associated with lower stage tumors and an apparently normal p53 gene, whereas low expression appeared to be associated with mutations in the p53 gene. However, some tumors displayed high levels of p21Cip1 even in the presence of presumed mutations in p53 (63, 65). It appears that both precursor lesions and carcinomas can display dysregulation in the expression of p21Cip1 (62, 65). We are not aware of any studies on the prognostic significance of p21Cip1, although its normal association with differentiation and its loss in high-grade tumors suggest that loss of p21Cip1 expression may be associated with a poor prognosis.

We have previously reported increased expression of cyclin D1 in human adenomatous polyps of the colon and in colon carcinomas (8). Recent studies indicate that this is also seen in the small polyps obtained from patients with polyposis coli or APC-deficient mice with polyposis coli (11). Therefore, increased expression of cyclin D1 can occur at an early stage of colon carcinogenesis. The present study confirms and extends these findings. Thus, in contrast to p27Kip1 (Fig. 1a), normal colonic mucosa expressed very low levels of cyclin D1, and there was a progressive increase in the expression of this protein in colonic polyps and colorectal carcinomas (Fig. 1d). There was a significant correlation between high expression of p27Kip1 and cyclin D1 in the adenomatous polyps and in the subset of carcinomas that had only nuclear p27Kip1 expression. This finding is reminiscent of our evidence that these two proteins appear to be coregulated in a subset of human cancer cell lines (46, 47, 50). A recent study (60) also found an association between levels of expression of cyclin D1 and p27Kip1 in primary human breast cancers. This association has also been seen not only in esophageal cancer cell lines (46, 66) but also in primary squamous carcinomas of the esophagus.

During the course of these studies, we observed that lymphocytes in the stroma of the normal colon mucosa and in the polyp and carcinoma samples displayed intense immunostaining for p27Kip1. Within lymphatic follicles, the germinal center showed intense Ki67 staining but no p27Kip1 staining, whereas in the peripheral zone, there was an inverse staining pattern (Fig. 2, C and D). Therefore, in contrast to normal colonic mucosa and colon carcinoma cells, proliferating lymphoid cells markedly down-regulate the expression of p27Kip1. Furthermore, the intensity of p27Kip1 staining in the lymphatic cells was usually much greater than in the positive colonic mucosal cells. Therefore, it would appear that the role of p27Kip1 in regulating withdrawal from the cell cycle varies between cell types. This conclusion is consistent with effects seen in control and p27Kip1-deficient mice (63, 64). In control mice, among various normal tissues, the thymus and spleen express the highest level of p27Kip1; and in the deficient mice, these organs show the greatest increase in size.

Finally, it is of interest to consider the findings in this study and in other studies on p27Kip1 expression in human cancers (54—56, 60, 61) within the broad context of growth control and carcinogenesis. What is the explanation for the apparent paradox (see Fig. 1a) that about 50% of adenomatous polyps and colorectal carcinomas of the colon express levels of an inhibitor of the cell cycle that are equal to or greater than those seen in normal colonic mucosa? Other recent studies have also seen relatively high levels of expression of p27Kip1 in a subset of colorectal and breast cancers (54—56, 60). In addition, as mentioned above, exponentially dividing cultures of human colon and breast cancer cell lines also frequently express high levels of this protein (46—50, 60). It is curious that another member of the Cip family of CKIs, p21Cip1, is also expressed at relatively high levels in some human cancers (67). On the basis of our in vitro studies with cell lines, we previously postulated the existence of feedback inhibitory loops between cyclin D or E and p27Kip1, the function of which is to maintain homeostatic control between positive- and negative-acting factors involved in cell cycle progression (46—50, 52). The association between high levels of cyclin D1 and p27Kip1 in adenomatous polyps and a subset of colon carcinomas seen in the present study, and also in previous studies on breast (60) and esophageal carcinomas (see above), is consistent with this hypothesis. This association was not, however, seen in all of the carcinomas, presumably because cyclin E (48—50) and other unknown factors can also influence the level of p27Kip1 expression. The function of p27Kip1 in the above-described homeostatic feedback loop might be to prevent potentially toxic effects of excessive cyclin D/Cdk or cyclin E/Cdk kinase activity. The coordinate expression of cyclin D1 and p27Kip1 might also be relevant to recent evidence that at low concentrations, members of the Cip family of CKIs may play a positive role by promoting the association of D-type cyclins with Cdk4, thus enhancing kinase activity, and they may also play a role in targeting cyclin D1 and Cdk4 to the nucleus (68).

The association of high p27Kip1 expression with low-grade tumors in this and in previously published studies (55, 60, 61) is consistent with evidence for a role of p27Kip1 in development and differentiation (40, 69—71), but the precise role that it plays is not apparent at the present time. We do not yet have follow-up data on the cases examined in this study, but other recent studies found that cases of colon, breast, or non-small cell lung cancer with low or absent p27Kip1 expression had a poor prognosis (54—56, 61). Perhaps this is because they have escaped the above-mentioned putative homeostatic feedback inhibitory function of p27Kip1 through further alterations in the complex circuitry that controls the cell cycle. Additional studies are required to elucidate the multiple factors that influence the levels of p27Kip1 expression in human cancer and whether the levels of expression of this protein, cyclin D1, or related cell cycle control proteins can be exploited in the therapy of specific cancers.

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5 Y. Doki, personal communication.
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