Morphological and Molecular Processes of Polyp Formation in ApcΔ716 Knockout Mice

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Abstract

Mutations in the human adenomatous polyposis coli (APC) gene are responsible for not only familial adenomatous polyposis but also many sporadic cancers of the digestive tract. Using homologous recombination in embryonic stem cells, we recently constructed Apc gene knockout mice that contained a truncation mutation at codon 716 (ApcΔ716). The heterozygous mice developed numerous intestinal polyps. All microadenomas dissected from nascent polyps had already lost the wild-type allele, indicating the loss of heterozygosity (M. Oshima et al., Proc. Natl. Acad. Sci. USA, 92: 4482—4486, 1995). We also demonstrated that cyclooxygenase 2 is induced in the polyps at an early stage and plays a key role in polyp development (M. Oshima et al., Cell 87: 803—809, 1996).

We have analyzed the process of polyp development in these mice both at morphological and molecular levels. A small intestinal microadenoma is initiated as an outpouching pouch in a single crypt and develops into the inner (lacteal) side of a neighboring villus forming a double-layer nascent polyp. The microadenoma then enlarges and gets folded inside the villus. When it fills the intravillous space, it expands downward and extends into adjoining villi, rather than rupturing into the intestinal lumen. During this course of development, the basement membrane remains intact, and the labeling index of the microadenoma cells is similar to that of the normal crypt epithelium. As in the crypt cells, neither transforming growth factor β1 nor its receptor type II is expressed in the microadenoma cells. No hot spot mutations in the K-ras gene are found in the microadenoma tissue during these early stages of polyp development. Essentially, the same results have been obtained for the colonic polyps as well. These results suggest that early adenomas in the ApcΔ716 polyps are very similar to the normal proliferating cells of the crypt except for the lack of directed migration along the crypt-villus axis.

Introduction

Mutations in the APC2 gene on chromosome 5q21 (1—4) are responsible for not only FAP but also many sporadic cancers of the digestive tract such as colorectal axis, stomach, and esophagus (5—7). The gene consists of 15 exons encoding a huge protein of about 2840 amino acids and several 5' noncoding exons. Exons 1—14 are relatively short, whereas exon 15 occupies three quarters of the whole coding region. Most FAP patients carry truncation mutations in the NH2-terminal half (8). Approximately 900 residues of the NH2-terminal of the APC protein contain proline-free blocks with heptad repeats of hydrophobic residues. Such a pattern is characteristic of α-helical coiled-coils and implicated in protein-protein interactions, suggesting its association with itself and/or with other cellular proteins (1, 3). In fact, a subdomain of the first 55 amino acids forms a stable, parallel helical dimer (9). Truncated APC proteins can associate with the wild-type protein in vivo as well (10). Accordingly, it was suggested that the truncated APC protein inactivates the wild-type protein in a dominant-negative manner. However, such a hypothesis was ruled out by experiments using transgenic mice that expressed third copy Apc minigenes with truncation mutations at high levels in the gut, because not a single polyp developed in any of these transgenic mice (11). Immunostaining studies showed that the wild-type, but not mutant, APC protein associates with the microtubule cytoskeleton (12, 13). A recent immunocytochemical analysis demonstrated localization of APC protein at filopodia at the leading edges of migrating cells, indicating that APC protein is involved in cell migration (14). In addition, forced expression of APC protein in transgenic mice induces disordered cell migration in the intestinal epithelium (15). Several mouse models of FAP were constructed either by chemical mutagenesis of the Apc gene (Min mouse, a truncation mutation at codon 850; Refs. 16 and 17) or by homologous recombination in embryonic stem cells (Apc1638 at codon 1638, Ref. 18; ApcΔ716 at codon 716, Ref. 19). In all of these mice, the homozygous mutants die in utero before 8 days postcoitum, whereas heterozygotes develop polyps in the small and large intestines. Interestingly, most polyps are found in the small intestine, although a relatively small but significant number of polyps develop in the colon, a phenotype different from human FAP. Two genes were reported to affect intestinal polyp formation in Min mice, i.e., DNA methyltransferase and the modifier of Min-I (Mom-I) genes (20, 21). The clinical relevance of these genes, however, remains to be investigated further.

On the other hand, we recently presented genetic and pharmacological evidence that the cyclooxygenase-2 gene plays a key role in polyp formation in ApcΔ716 knockout mice (22).

In this report, we describe the results of morphological, immunohistochemical, and genetic analyses of the processes of polyp formation in the ApcΔ716 mice and discuss the molecular mechanism behind them.

Materials and Methods

ApcΔ716 Knockout Mice. Construction and basic characterization of ApcΔ716 knockout mice have been described previously (19). The C57BL/6-ApcΔ716 pedigree is maintained at Banyu Tsukuba Research Institute (Merck) by crossing male ApcΔ716 heterozygotes with C57BL/6N females.

Morphological Analysis of Polyposis. Dissection and analysis of the intestinal/colonic polyps under a dissection microscope and their histological investigation have been described earlier (19, 22—24). Briefly, the entire small intestinal tract and colon were filled with 10% formaldehyde-PBS, opened longitudinally, and examined under a dissection microscope. For histological examinations, fixed intestines were embedded in paraffin and sectioned at 5-μm thickness. Sections were stained with H&E or toluidine blue.

Morphological Analysis of ACF. A methylene blue tattoo method (25) was used. Briefly, colon samples were opened longitudinally and fixed in 10% formaldehyde-PBS. The fixed samples were stained with 0.2% methylene blue-PBS and examined under a dissecting microscope.
**Results and Discussion**

We previously constructed and analyzed the Apc<sup>Δ716</sup> knockout mice and found nascent polyps that consisted of a layer of microadenoma growing inside the normal epithelium of a single villus (Ref. 19; Fig. 1, A, D, and G). When the microadenoma inside such a nascent polyp proliferated further, the adenoma tissue was folded with an increased surface area inside the normal villous epithelium (Fig. 1, B, E, and H). Upon further expansion of the adenoma, however, it did not rupture into the intestinal lumen but somehow expanded into the adjoining villi through the inner (i.e., mucosal) side, leaving the normal villous epithelium intact. As shown in Fig. 1, C, F, and I, larger polyps had an appearance of multiple villi stuck together, with the normal epithelium of several villi still covering the adenoma tissue. These dissection micrographs and histological pictures are summarized schematically in Fig. 2 (see “Discussion”). As we described earlier, these polyps were histologically classified as adenomas (19).

Because of its relevance to human FAP and colon cancer, it is important to investigate the development of colonic polyps in the Apc<sup>Δ716</sup> mice. As shown in Fig. 4, we found histological pictures that suggest a similar mechanism of polyp formation to that in the small
proposed that human colorectal tumorigenesis proceeds through a series of genetic alterations involving oncogenes (ras), DNA hypomethylation, and tumor suppressor genes such as APC, DCC, and/or p53 (28, 29). Accordingly, we determined the K-ras gene (Kras2) sequence in the early stage intestinal polyps from the Apc\(^{716}\) heterozygotes. Among five microadenoma samples we tested, none of them showed mutations in the hot spots corresponding to codon 12 or 61 of Kras2 (data not shown).

Several lines of evidence indicated that some of “ACF” of the colon may be a precancerous lesion. These cryptal lesions, originally observed in carcinogen-treated mice or rats and later confirmed in humans as well, are distinguished by their increased size, thicker epithelial lining, and increased pericryptal zone (30). Human ACF can be classified as either dysplastic or nondysplastic, with the latter far outnumbering the former (31, 32). Moreover, K-RAS (KRAS2) muta-
tions were reported to be remarkably common in small nondysplastic ACF (31–33). Accordingly, we made a thorough search for ACF in the Apc<sup>Δ716</sup> mice by the methylene blue tattoo method (25). However, we could not find even a single ACF in any mice tested (n = 15; data not shown).

To investigate the process of tumorigenesis in Apc<sup>Δ716</sup> knockout mice, we determined the labeling indices for the polyp adenoma cells and compared them with those in the normal small intestinal crypt or colonic gland proliferation zone. As shown in Fig. 5A, the labeling index of the small intestinal microadenomas was 28.9% ± 4.8% (SD), and essentially the same as that of the normal crypts (i.e., 27.6% ± 3.3%). On the other hand, the labeling index of the colonic adenomas was 20.6% ± 0.05%, and significantly lower than that of the normal colonic gland proliferation zone (i.e., 27.9% ± 5.8%). Typical histological specimens of the BrdUrd labeling and PCNA staining are shown in Fig. 5, C–J.

Accumulating evidence indicates that TGF-β is induced in the intestinal epithelial cells in an autoregulated manner, inhibits their proliferative activity, and promotes differentiation (Ref. 34; reviewed in Ref. 35). Although malignant colon adenocarcinomas are resistant to TGF-β, nontumorigenic early adenomas are sensitive to the growth-inhibitory effects of TGF-β (36). The effects of TGF-β on the intestinal and colonic epithelia are mediated through the TGF-β receptor type I and type II complex (35). To assess the role of TGF-β and TGF-β receptors in Apc<sup>Δ716</sup> intestinal polyposis, we determined the expression of TGF-β1 and TGF-βRII in the polyp microadenomas as well as in the normal intestinal epithelium (Fig. 6). In the small intestine, TGF-β1 was expressed only in the villus epithelium but not in the crypt cells (Fig. 6A), whereas in the colon, TGF-β1 signals were detected abundantly in the differentiated epithelium on the luminal one-third to one-half of the crypt (Fig. 6C). In the Apc<sup>Δ716</sup> polyps, the adenomas remained unstained both in the small intestine and colon (Fig. 6, E and G, respectively). When specimens were stained for TGF-βRII, essentially the same results were obtained as for TGF-β1, i.e., only the villus epithelium of the small intestine and luminal one-third to one-half of the colonic crypt epithelium were stained in the normal tissues (Fig. 6, B and D, respectively), whereas the polyp adenomas of the small intestine and colon remained unstained (Fig. 6, F and H, respectively). Immunohistochemical staining of early stage polyps showed a marked contrast of the stained normal villus epithelium covering the microadenoma cells that remained unstained (Fig. 6, I and J). Specificity of these antibodies were confirmed by absorption experiments using synthetic peptide antigens (data not shown; see "Materials and Methods"). In conclusion, not only the crypt cells but also the polyp microadenomas were negative immunohistochemically regarding TGF-β1 and TGF-βRII.

The heterozygous Apc<sup>Δ716</sup> knockout mice develop numerous intestinal and colonic polyps (19). Their polyp numbers were three to five times higher than those in the Min mice (Apc<sup>Min</sup>; Ref. 16), which enabled us to analyze polyps at various stages of development in detail. The process of polyp development we have described here is totally consistent with a function of the Apc gene product proposed recently. Evidence has been presented that APC protein is involved in cell migration (14), whereas forced expression of APC protein in transgenic mice induces disordered cell migration in the intestinal epithelium (15). It is likely that APC protein is needed for the normal migration of the proliferation zone cells along the crypt-villus axis of the small intestine. When the proliferation zone cells lack the full-length wild-type APC protein, they are likely to form an outpocketing pouch rather than migrating toward the tip of the villus. In this context, the APC/Apc gene does not appear to be an antioncogene or tumor suppressor gene, such as the RB or p53 gene. In fact, the labeling indices for the microadenoma cells are not higher than the crypt proliferation zone cells (Fig. 5, A and B). In a thorough study of rectal biopsies from 400 patients, Risio et al. (37) presented compelling evidence that proliferation of the epithelial cells is related to the size and chronology of preneoplastic lesions, and that labeling index values rose progressively from adenoma to adenocarcinoma. Adenomas with a diameter of <1 cm were not accompanied by changes in the mucosal labeling index. Our data are consistent with their report. Another interesting phenomenon we have found is that the expanding adenoma tissue does not rupture through the covering normal villous epithelium into the intestinal lumen, but rather extends into adjoining villi from the mucosal side. Even in relatively advanced polyps that consist of multiple villi, the normal villous epithelium was still conserved (Figs. 1f and 2H). The molecular mechanism that controls such a growth pattern of the adenoma tissue remains to be investigated.

Because the colon lacks villi, it is not easy to locate early colonic
polyps under a dissection microscope. However, serial sectioning analysis enabled us to detect colonic polyps at relatively early stages (Fig. 4, A and B). These polyps showed a morphology that suggests a similar initial mechanism to that in the small intestine: formation of outpocketing pouch or buds. A similar outpocketing pouch has been reported as one of the two initial mechanisms of colon cancer development in chemical carcinogen-treated mice (38). Development of an outpocketing pouch or a bud into a single-gland adenoma was reported in human FAP as well (39), and our observation of colonic polyp development in the Apc<sup>5716</sup> mice is consistent with these reports. Moreover, DNA extracted from these microadenomas dissected from sections also showed Apc gene LOH (Fig. 4D), suggesting that the LOH is important in triggering polyp formation in the colon as in the small intestine (19). Although “ACF” have been reported as preneoplastic lesions, we did not find any ACF in the Apc<sup>5716</sup> colon. Accordingly, ACF are not the direct precursors of the colonic polyps in the Apc<sup>5716</sup> heterozygous mice.

It has been demonstrated that expressions of TGF-β and its type II receptor are regulated in a concerted manner, showing the same spatio-temporal expression pattern, e.g., in the embryo (40) and skin (41). Here we have shown their concerted expression in the small and large intestinal mucosa, i.e., the expression of both TGF-β1 and TGF-βRII in the differentiated epithelium but not in the undifferentiated proliferation zone cells. Moreover, the polyp adenomas do not express either molecules at levels detectable by immunohistochemistry. These results, together with the above data of labeling indices and lack of K-ras hot spot mutations, strongly suggest that the early adenoma cells have very similar charac-

Fig. 5. Labeling indices of the polyp adenoma cells compared with those of the normal mucosal epithelia of the small intestine and colon in Apc<sup>5716</sup> knockout mice. A and B, labeling indices for the small intestine and colon, respectively. A, Crypt, normal epithelium of the small intestinal crypt. B, Prolif. Z., proliferation zone of the colonic gland. Polyp, polyp adenoma epithelium, excluding the interstitial cells. C and E, BrdUrd (BrdU) labeling of proliferating cells in a small intestinal section containing polyps. D and F, H&E (HE)-stained serial sections adjoining those in C and E, respectively. G, BrdUrd (BrdU) labeling of a colonic mucosal section containing an early adenoma. * an early adenoma. H, an H&E (HE)-stained serial section adjoining that in G. I and J, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) in small intestinal mucosal sections that contain early polyps. Arrows, PCNA-labeled cells. Bars: C–F and I, 200 μm; G, H, and J, 100 μm.

Fig. 6. Immunohistochemical staining for TGF-β1 and TGF-βRII in normal small intestinal and colonic epithelia, compared with those in sections containing small intestinal polyps and colonic tumors in Apc<sup>5716</sup> heterozygotes. A and B, normal small intestinal epithelium. C and D, normal colonic epithelium. E and F, sections of the small intestine containing polyps. G and H, sections of the colon containing adenomas. Arrows, adenoma tissues negative for TGF-β1 (G) and TGF-βRII (H). I and J, sections of the small intestine containing a nascent (I) and univillous polyps (J). A, C, E, and G, staining for TGF-β1. B, D, F, H, I, and J, staining for TGF-βRII. Bars: 100 μm for all frames.
teristics with those of the proliferation zone of the normal mucosa. To investigate the role of TGF-βRII in development, we recently knocked out its gene in the mouse (42). Although the heterozygotes were developmentally normal, all homozygous mutants died in utero around 10.5 days of gestation because of defects in yolk sac hematopoiesis. To study the role of TGF-βRII in the intestinal mucosa, heterozygous mutation was introduced into the Apc<sup>–/–</sup> knockout mice, and double heterozygotes were constructed. However, there was no change in the number and size of the intestinal polyps (data not shown). These data suggest that either the haploid amount of TGF-βRII protein is sufficient for the normal function of the intestinal mucosa, or TGF-βRII is not playing any significant role in polypl formation in Apc<sup>–/–</sup> knockout mice.

Recently, overexpression of cyclin D1 and cyclin-dependent kinase 4 has been demonstrated in the intestinal adenomas from the Min mice and human FAP (43). Cyclin D1 expression was restricted to the adenoma cells and detected even in low-dysplasia adenomas. It is possible that these may be early intracellular events following Apc gene LOH. It would be interesting to investigate its relationship to cyclooxygenase-2 induction (22).

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References


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