Colossal Crypts Bordering Colon Adenomas in *Apc*^{Min} Mice Express Full-Length Apc

Matthew Bjerknes and Hazel Cheng

From the Department of Anatomy and Cell Biology, University of Toronto, Toronto, Ontario, Canada

Enlarged but nondysplastic crypts are frequently observed at the margins of colon tumors, forming what has been called a transitional epithelium. It is now thought that this is a reactive state and not a preneoplastic condition as previously suggested. We have used the mouse familial adenomatous polyposis model, *Apc*^{Min}, to study these abnormal adenoma-associated crypts. We report that these non-dysplastic crypts are enormous (as much as 10 times normal length) and branch more frequently than normal crypts. They express wild-type Apc protein and display the wild-type *Apc* allele. We conclude that the colossal crypts at adenoma margins have normal *Apc* gene function, consistent with the suggestion that their phenotype is a reactive state. The cause remains an open question, but the dramatic epithelial response hints at the presence of potent epithelial trophic factors in the vicinity of colon tumors. (Am J Pathol 1999, 154:1831–1834)

It has long been recognized that the colonic epithelium at the margins of adenocarcinomas, although not neoplastic, is not normal. The crypts in this transitional region are enlarged and often branched.[1–5] However, as a similar state is observed at the margins of an array of lesions, it is now believed that it represents a reactive state and not a preneoplastic condition[6,7] and has since been largely ignored. We have noticed in studies of a mouse model of familial adenomatous polyposis[8] that although the dysplastic epithelium forming the bulk of adenomas is Apc negative, there is often a layer of Apc-positive nondysplastic crypts underlying the tumor.[9] These Apc-positive crypts are often cut in cross section when the tumor is sectioned orthogonally to the surface. This observation raised questions about the relationship of the Apc-positive crypts to the overlying tumor and to the surrounding normal epithelium. We show here that they represent greatly elongated crypts, with normal Apc gene function, that extend under the tumor from the margins.

Materials and Methods

Isolation of Intact Epithelium and Scanning Electron Microscopy

Fifteen week-old *Apc*^{Min} mice (Jackson Laboratories, Bar Harbor, ME) were used. Intact epithelium was isolated, from small segments of colon each containing an adenoma, by intraventricular perfusion of 30 mmol/L EDTA in PBS followed by vibration into 4% paraformaldehyde in PBS.[10] After fixation for 30 minutes on ice, the isolated epithelium was washed in PBS and then rinsed and stored in methanol at −20°C. The colon adenomas and adjacent mucosa were dissected from the vibrated segments and fixed for 30 minutes in 2.5% glutaraldehyde in PBS. The adenomas were then processed for scanning electron microscopy (SEM) by post-fixing in 1% osmium tetroxide in 0.1 mol/L cacodylate HCl buffer, pH 7.4, and then dehydrated through a graded series of ethanol, critical-point dried,[11] and sputter coated with gold palladium.

Immunofluorescence

Colossal crypts were microdissected from the isolated epithelium, rinsed in 1% Nonidet P-40 (NP40) in PBS, freeze-thawed at −20°C to increase permeability, rinsed in 1% NP40, and blocked at room temperature for 1 hour in PBS containing 10% newborn calf serum and 1% NP40. The crypts were incubated overnight at 4°C in rabbit polyclonal antibodies specific for the carboxy terminus of Apc protein (Santa Cruz Biotechnology, Santa Cruz, CA), washed at room temperature with 1% NP40 for 3 hours, incubated overnight at 4°C with Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and then washed with 1% NP40. Controls, which were negative, included omission of the primary anti-Apc antibody, omission of both antibodies, and Apc peptide competition.

Cryosections from colon adenomas were incubated with the anti-Apc antibodies and then stained with Cy3-conjugated goat anti-rabbit IgG as described.[9]
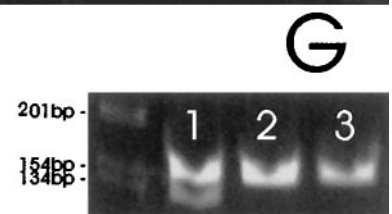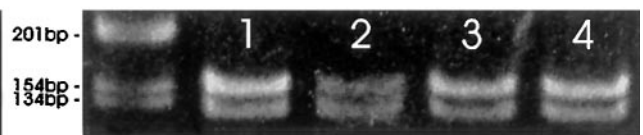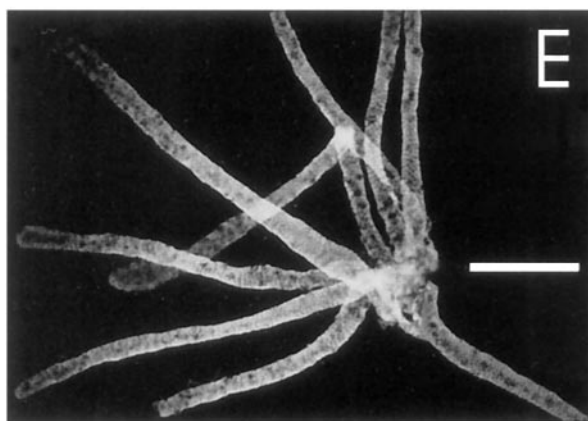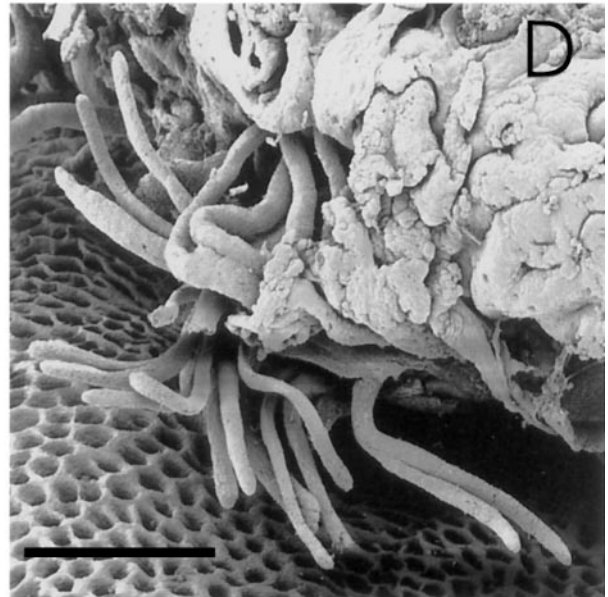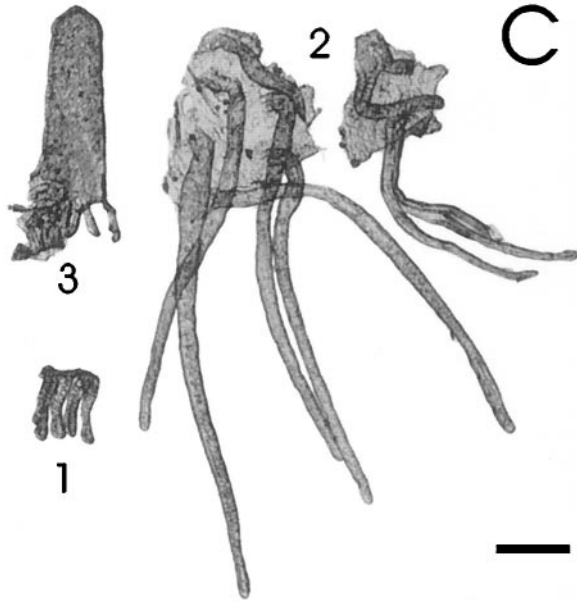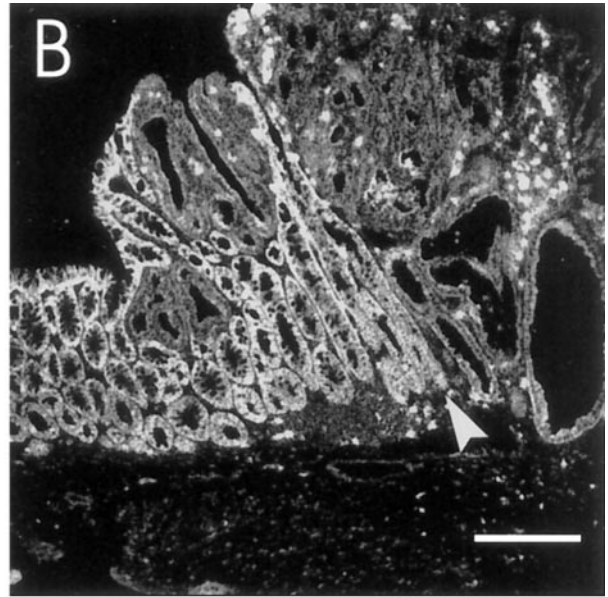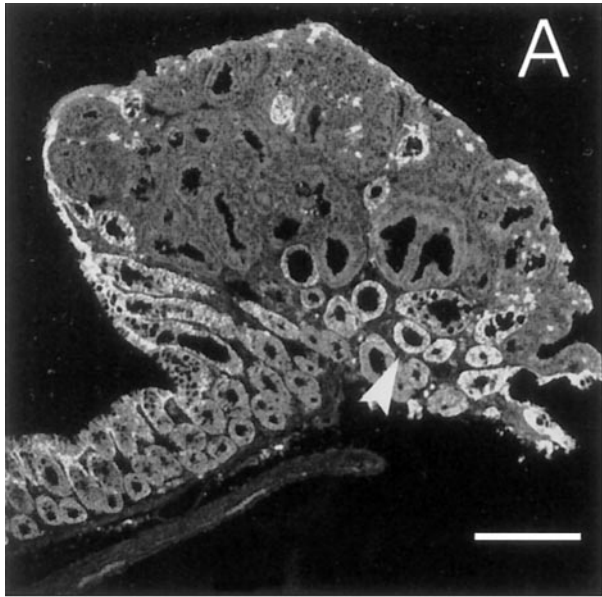
Testing for Loss of Apc Heterozygosity by Polymerase Chain Reaction

Single colossal crypts were microdissected from the isolated epithelium. Small sheets of dysplastic epithelium were microdissected from the adenomas. The microdissected tissues were placed in individual polymerase chain reaction (PCR) tubes and digested overnight at 50°C in 10 μl of lysis buffer containing 1 mg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN), 10 mmol/L Tris/HCL (pH 8), 50 mmol/L KCl, 0.45% NP40, and 0.45% Tween 20. The next morning the proteinase K was heat inactivated (10 minutes at 95°C), the tubes were centrifuged for 5 minutes, and the supernatant was transferred to new tubes. Loss of heterozygosity (LOH) of the *Apc* gene was checked using PCR.[12] Briefly, after digestion with *Hin*dIII the *Apc*[Min] allele PCR product generates a 144-bp fragment whereas the wild-type allele PCR product generates a 123-bp fragment. Thus, two DNA bands appear on gels (12% nondenaturing polyacrylamide gel electrophoresis) from phenotypically normal tissue from *Apc*[Min] mice. In contrast, as the vast majority of adenomas in *Apc*[Min] mice result from cells that have lost the wild-type allele,[9,12] the dysplastic epithelium from adenoma displays LOH, and only one band is evident from the *Apc*[Min] allele.

Branching Crypt Frequency

Crypts from the epithelium isolated from four of the *Apc*[Min] mice were randomly selected and mounted on slides. The frequency of crypts that were branching was determined using an oil immersion 100× objective. During the scoring, colossal crypts were identified by their size (usually 5 to 10 times longer than normal) and were recorded separately. The frequencies of branching normal and colossal crypts were compared with a two-tailed test for the equality of binomial means.[13]

Results and Discussion

Sections through colon adenomas from *Apc*[Min] mice often display a layer of nondysplastic, Apc-expressing crypts underlying the dysplastic tissue, but the relationship of these Apc-positive crypts to surrounding normal tissue was unclear. Some sections suggest that the crypts bordering adenomas are enlarged and extend under and sometimes into the margins of the tumor (Figure 1, A and B). When isolated, these crypts were found to be enormous, ranging from 2 to 10 times longer than normal crypts (Figure 1, C and D). The branching frequency of these colossal crypts (11.8%; 12 of 102 crypts scored were branching) was significantly higher ($P = 10^{-5}$) than that of normal crypts (2.4%; 99 of 4048 normal crypts scored were branching).

Crypt branching is the usual means of crypt replication with consequent epithelial growth.[14,15] The most appealing interpretation of the observation of an increased frequency of branching among the colossal crypts is that they are cycling faster than are normal crypts, potentially adding to tumor bulk with time. However, simply observing an increase in branching frequency is not sufficient to prove that the crypts are cycling faster. For example, if colossal crypts require a longer time to complete the branching process, due perhaps to their large size, then an increased proportion of branching crypts would be expected even if normal and colossal crypts have similar crypt cycle times.

Abnormalities in the frequency of branching non-neoplastic crypts have been reported in murine (*Apc*[Min]) and human (familial adenomatous polyposis) APC± colon.[16–18] It seems unlikely that this effect is directly related to the increased branching frequency among the colossal crypts found at tumor margins as the general effect is small and age dependent in mouse colon.[17,18] In humans, dysplastic crypts have a greatly increased branching frequency in comparison with non-neoplastic crypts.[16–18] Others have reported a 19-fold increase in branching rates of non-neoplastic crypts in these patients,[18] although we found no evidence of this[17] when obviously dysplastic crypts were excluded from counts (dysplastic crypts are frequent even when care is taken to sample only regions containing no macroscopic lesions).

Unusual tubular structures have also been observed near the margins of a wide variety of ulcers.[19] These highly contorted structures grow out from the base of neighboring crypts and are composed of a specialized nonproliferative cell lineage.[19] In contrast, the colossal crypts are independent structures with a well defined crypt base indicating that they are probably a distinct phenomenon.

Figure 1. Demonstration of the presence of colossal crypts at the margin of colon adenomas from *Apc*[Min] mice and their *Apc* status. All scale bars represent 250 μm. **A** and **B**: Laser scanning confocal micrographs of frozen sections through colon adenomas from *Apc*[Min] mice. The sections have been stained with an antibody specific for the carboxy terminus of Apc. In **A**, note the absence of staining in the dysplastic tissue and the layer of Apc-positive crypts underlying the tumor (**arrowhead**). There are also some enlarged crypts, sectioned in a more longitudinal plane, at the left tumor margin. In **B**, note the large Apc-positive branching nondysplastic crypt at the tumor margin (**arrowhead**). **C**: Photomicrograph of intestinal epithelium isolated from an *Apc*[Min] mouse. A group of crypts from normal colon is labeled 1. Compare them with the two sets of colossal crypts from the margins of an adenoma, labeled 2. Note the branching colossal crypt on the right. A normal crypt-villus unit isolated from proximal jejunum, labeled 3, is shown for size comparison. **D**: Scanning electron micrograph showing the base of a large adenoma from the colon (note the honeycomb-like array of vacant pits in the lamina propria, from which crypts have been extracted by the isolation procedure). A few colossal crypts, which have been extracted from the margins of the tumor, remain attached to the tumor by surface epithelium. Many of the crypts are branched. The crypts shown are ~500 μm long (approximately double the normal crypt height), although they appear smaller in this view due to foreshortening and partial obstruction of the crypt tops. Dysplastic epithelium is visible in the upper right corner. **E** and **F**: Fluorescence micrographs of isolated colossal (**E**) and normal crypts (**F**) stained for Apc protein with an antibody specific for the carboxy terminus. This demonstrates that the cells in colossal crypts are making full-length Apc. The figures are at the same magnification. **G**: Ethidium-bromide-stained gel showing PCR product from single colossal crypts (**lanes 1** and **2**) and groups of normal crypts (**lanes 3** and **4**). Note the presence of both wild-type (123-bp) and *Apc*[Min] (144-bp) alleles in both colossal and normal crypts. This confirms the antibody results presented in **E** and **F**. **H**: Demonstration of the loss of heterozygosity that occurs in dysplastic epithelium from *Apc*[Min] mice. **Lane 1** shows PCR product from normal epithelium (with both *Apc* alleles) whereas **lanes 2** and **3** show product from isolated dysplastic epithelium from adenomas (demonstrating only the *Apc*[Min] allele).

Dysplastic epithelium from colon adenomas displays LOH of the *Apc* locus (Figure 1H), as is usually the case in tumors from *Apc*[Min] mice.[9,12] The dysplastic epithelium also does not stain with the anti-Apc antibody specific for the carboxy terminus of Apc protein (Figure 1, A and B). In contrast, the colossal crypts do stain (Figure 1, A, B, and E) and have wild-type *Apc* alleles (Figure 1G) indicating normal *Apc* gene function. Furthermore, the Apc staining pattern, which normally increases in intensity from crypt base to top (Figure 1F[20]), appears normal in colossal crypts (Figure 1E), suggesting a normal differentiation program in the colossal crypt cells.

As loss of function of the *Apc* gene, usually by LOH,[9,12] is the cause of dysplasia in this mouse model, our findings are consistent with previous conclusions that the nondysplastic colossal crypts found on the borders of tumors are indeed reactive and are not directly involved in tumorigenesis,[6,7] although they probably add to tumor bulk. This raises the question of the signal inducing the exceptional size and branching frequency of these crypts. Is it a growth factor originating from the tumor, or could it be a cytokine released as a result of immune interactions in the tumor? The response of the epithelium is so dramatic that the underlying cause should be investigated, as it likely involves potent epithelial trophic factors that may have clinical application, for example, in inducing epithelial repair.

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