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ARTICLE

Patterns of Proliferative Changes in Crypts Bordering Colonic Tumors: Zonal Histology and Cell Cycle Marker Expression

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Proliferative crypt changes have been noted in mucosa bordering colonic carcinomas, but their biological significance is disputed. We anticipated that zonal patterning of histological changes and cell cycle marker expression would provide clues to the pathogenesis of these border changes. 81 specimens were examined including carcinomas, adenomatours polyps, adenomas with early carcinoma, flat adenomas and aberrant crypt foci. The spatial distribution and frequency of micro-architectural features, and mucosal thickness were determined in a border domain of 150–300 sequential crypts/specimen. Immunocytochemical expression of Ki67 and p53 antigens in crypts also was semi-quantitatively examined. We found that in 100% of carcinomas two histologically abnormal zones (Proximate and Middle) separated tumor from normal mucosa. Differences in the feature frequency between zones were statistically significant ($p<0.05$). Both zones showed mild increases in crypt cell expression of Ki67, with a statistically significant relationship to zonal patterning ($p<0.005$). Weak expression of p53 only appeared in rare cells. Crypt elongation with mucosal thickening (1.9x normal, $p<0.001$) in the Proximate and Middle zones distinguished carcinomas from border changes in all benign lesions, except flat adenomas. Since this change occurs in all cases of carcinoma, there is no correlation with tumor stage or grade. Also in carcinomas, elaborate complexes of attached crypts (*connected crypt structures)* were characteristic of the Middle zone, so that proximate zone was always architecturally simpler. We conclude, that despite continuous carcinoma growth, the invaded border mucosa maintains a prototypical zonal organization of molecular and histological crypt changes. This spatially organized reaction pattern is likely to reflect an interplay between regulated growth and destructive processes in response to advancing carcinoma. Compared to the edges of benign colonic tumors, the edges of carcinomas are distinctive and consistent enough to be diagnostically useful. (Pathology Oncology Research Vol 5, No 4, 297–303, 1999)

Keywords: colorectal carcinoma, colonic polyps, aberrant crypt foci, crypt replication cycles

Introduction

The progressive invasion by colorectal carcinoma of its bordering mucosa produces a laterally advancing wave of crypt destruction and reaction. Indications of abnormal crypt proliferation in this edge mucosa have been noted both histologically and immunocytochemically, but there is still no consensus regarding the biological interpretations of these changes. Aside from a remnant of the precursor lesion,[1,2] they could presage independent growth potential indicative of early neoplastic events[3-8] due to oncogenic mutations.[9] Alternatively, these changes may merely represent regulated, growth responses to the progressively advancing tumor.[10-13] The latter could be mediated by a number of factors including mechanical, vascular,[12] inflammatory[11] and humoral processes.[13]

Our approach to this problem was twofold. First, to examine the edge mucosa for zonal patterning of both histologic and molecular proliferative features. Second, to compare these changes to benign mucosal tumors, representing a possible progressive sequence leading to malignancy,[13] such as adenomatous polyps and aberrant crypt foci. The latter are putative pre-neoplastic lesions that often result in small elevations of the mucosa focally.[14-18] The rationale for this design is that zonal patterning often provides an indication of the pathogenetic sequence

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involved in pathological changes. A further implication of highly consistent zonal patterning across cases is that the proliferative changes would be unlikely to reflect oncogenic mutations, which are rare events.[19] A comparison of benign lesions was expected to highlight features unique to carcinomas, and perhaps detect the stages in the progression towards carcinoma when the changes appear.

In examining the molecular manifestations of altered proliferation we used Ki67, an antigen expressed in nuclei during all phases of the cell cycle except G0, and is a widely used marker for proliferating cells.[20] In normal crypts the proliferating cell population is topologically restricted to the lower third of the crypt long axis.[21,22] Neoplasia is often reflected in loss of this topological organization with diffuse expression, accompanied by a substantially increased frequency of proliferating cells.[4,20] In colonic carcinomas, p53 is often mutated leading to readily detectable intracellular accumulation.[21] It may also be up-regulated in normal tissues following local DNA damage.[21]

Materials and Methods

Tissue samples

Eighty one specimens were obtained from archival blocks or fixed gross specimens of the Department of Pathology, Hasharon Hospital, Rabin Medical Center-Golda Campus, Petach Tikvah, Israel. They were selected based on the microscopic suitability for zonal analysis, as described below. The material included adenocarcinomas (35), adenomatous polyps (20**),** aberrant crypt foci, ACF (15**),** polyps with minimally invasive carcinoma (3), flat adenomas (5), and controls without a lesion (3). Patients with carcinoma represented both sexes, aged 51 to 84, and had flat, exo- and endophytic lesions from all regions of the colon. Representative tumors from all histopathological grades and clinical stages Duke's B–D were esamined. Remote metastases were identified with radioisotopic imaging performed on all patients. Polyps were tubular, villous or mixed adenomas, and represented a range of dysplastic changes from mild to severe. The examples with focally invasive (early) carcinoma, contained small zones extending into the stalk, submucosa or muscularis propria, but not involving the lateral margins. Flat adenomas were non-polypoid zones of tubular-type (adenomatous) glandular elements with moderate to severe dysplasia, focally extending through the depth of the mucosa and laterally through the superficial depth compartment; one case was focally invasive. The ACF were sampled from archived formalin fixed gross resection specimens. They were identified by methylene blue staining of the unembedded mucosal surface, and marked with India ink under the microscope to enable blocking;[27] four ACF were incidental findings in blocks of resection margins. Both dysplastic and non-dysplastic ACF were examined. Among the controls, one was a case of intra-abdominal fat necrosis following abdominal trauma in a 40 year old, while the other two were remote margins in a carcinoma resection.

For the zonal examinations, one block from each specimen was used which included the lesion in continuity with adjacent mucosa. The criteria for selection of a block were as follows: mostly perpendicular orientation of crypts bordering the lesion; viable tumor adjacent to border crypts; identifiable tumor-mucosal interface, i.e. the beginning of an uninterrupted series of crypts extending away from the tumor. All carcinoma edge blocks included approximately 0.5 cm of adjacent mucosa, containing at least 150 crypts; usually over 300.

Fresh tissue is routinely fixed in 10% buffered formalin, embedded in paraffin, cut at 5μ and stained with hematoxylin and eosin.

Immunocytochemistry

Mouse anti-human monoclonal antibodies (IgG1) against Ki67 (clone MIB1, Zymed Laboratories, So. San Francisco, CA was utilized undiluted, according to suppliers recommendations. Following antigen retrieval,[32] slides were incubated for one hour at room temperature with the primary antibody. A mouse anti-human antibody to cellular phosphoprotein p53 (Zymed) was also utilized. Detection utilized an ABC system according to suppliers instructions (Zymed), with AEC as the chromogen, and Mayer's hematoxylin as counterstain. An irrelevant antibody of the same isotype served as a negative control, and intrinsic tissue elements, e.g. tumor and lymphoid aggregates, were the positive controls. Replications of several lesions gave consistent results.

Examination

The border mucosa, as defined above, was examined microscopically for zonal patterns of crypt changes. All slides were scored for the presence or absence of the histological changes noted in *Table 1*, and their zonal distribution. In addition, crypts were scored for the presence, and degree of dysplasia, based on previously established criteria.[33] Using a 14 x 14 ocular grid, calibrated with a Zeiss stage micrometer, the thickness of the proximate zone and distant zones were measured at points of approximate vertical sectioning of the mucosa in 10 cases.

Immunocytochemical preparations of carcinomas stained for Ki67 were examined in two ways. In the first, for each case, all histological zones were globally rated for the presence or absence (+/-) of "expanded *topology*" of immunoreactive cells. In normal mucosa, it is extremely uncommon for more than a rare Ki67 immunoreactive cell to occur within the superficial half of the mucosa, and crypts normally contain very few (<10%) immunoreactive cells. Internal

controls were provided by the distant zone, and external controls derived from mucosa remote from tumor or from patients without known tumor. For the second method, 10 well stained cases were randomly selected. For this group *frequency* of immunoreactive cells was scored in each of three depth compartments for each zone. A scale of 1+ to 3+ was used, standardized as follows: 1+, <10%; 2+, 20–40%; 3+>50%. Approximately 20 consecutive proximate crypts per zone were scored, except for the zone immediately adjacent to tumor, which often contained fewer crypts. Because of minimal expression, p53 antigen was qualitatively assessed in relation to zones and depth compartments.

Table 1. **Zonal histological changes in border crypts of colonic tumors**

		% Cases Showing Change				
Zone	*Histology*	*CRC[a] (35[b])*	*AP[a] (20)*	*ACF[a] (15)*	*MIA[a] (8)*	*CTL (3)*
P[a]	Long curved crypts	77*	0	0	88*	–
	CCS	23*	100	66	100	–
	Mucosal atrophy	0*	45	0	13	–
	Mild dysplasia[c]	23	0	0	0	–
M[a]	Elongated CCS[a]	100*	–	–	–	-
Da	NL / occasional CCS	73	100	60	100	100

a – CRC – colorectal carcinoma, AP – adenomatous (tubular or villous) polyp; ACF – aberrant crypt foci; MIA – minimally invasive or infiltrative adenomas (flat and polypoid); CCS – complex crypt structures; P – proximate zone; M – middle zone; D – distant zone; NL – normal; CTL – control.
b – ( ) = total number of samples; c – mild in all cases; * – statistically significant difference compared to same feature in AP, Chi-square, $p<0.05$.

Statistical analysis

The Chi-square test was used to examine the following data sets from carcinomas for statistical significance: the frequency of expanded topology versus the four immunocytochemically defined zones (DF=3); the frequency of expanded topology for each pair of zones (DF=1). The results of the semi-quantitative analysis of the frequency of stained cells ($n_1n_2 = 10$) were examined with the non-parametric Mann-Whitney U test with regard to zonal differences for Ki67, and depth compartmental differences/zone. Comparison of mucosal thickness between zones also utilized the U test.

Results

Zonally distributed histological alterations characterized the crypts bordering each type of lesion. Although certain crypt changes appeared in all lesion types, distinguishing features also were observed (*Table 1*). Enhanced Ki67 immunocytochemical expression, in the crypt long axis, was zonally patterned in carcinomas as well (*Tables 2,3*). For carcinomas, the principal changes were universally observed, and consequently were independent of tumor grade or stage.

Histological Findings

Carcinoma – The mucosa at the lateral margin of carcinomas could be divided into three zones (*Table 1, Figures 1,2*) based on histological features. The most proximate zone (**P**), was defined as crypts in direct contact with tumor either laterally or inferiorly. This zone was also recognizable because the tumor had destroyed the muscularis mucosa in most instances. Zone P crypts were elongated, usually non-branched, and arranged as oval profiles (*Figure 3*). The mucosa in zone P was thickened and measured 1356 ± 208 μ, compared to 716 ± 112 μ in the distant zone ($p<.001$, Mann–Whitney U test, $n_1n_2 = 10$). Qualitatively, the areal density of crypts was lower than in more lateral mucosa.

The next more lateral area was the middle zone (**M**), and in all cases was the most distinctive. It featured thickened mucosa due to elongated, crowded crypts that often formed elaborate complexes of interconnected crypts, referred to hereafter as connected crypt structures or CCSs. Frequently, the epithelium showed mild hyperplasia, and a marked increase in goblet cells superficially (*Figures 2, 4,5*). The

Table 2. **Frequency of expanded topology for Ki67 in carcinoma per Zone[a]**

Zone	*%Cases*	*Statistical Analysis*			
		Zone P[b]	*Zone Mp*	*Zone Md*	*Zone D*
P	94	–	ns	ns	<0.001[c]
Mp	87	ns	–	ns	<0.001
Md	73	ns	ns	–	<0.001
D	11	<0.001	<0.001	<0.001	–

a – Percent of cases showing expanded topology of Ki67 expression above the normal basal compartment for each zone. Statistical comparisons of each pair of zones is given in the four rightmost rows. Each statistical data cell represents the significance of one pair of zones defined by its x-axis and y-axis labels. Chi-square, DF=1, n=18.
b – P-proximate to tumor; Mp- middle zone, proximate portion; Md-middle zone, distant portion; D-distant zone;
c – p value: statistically significant; ns, differences were non-significant.

Table 3. **Frequency of Ki67 stained cells as a function of depth and Zone[a]**

Depth Compartment	*Zone P[b]*	*Zone M*	*Zone D*
Upper	0.4 ± 0.5	0	0
Mid	1.6 ± 0.7	0.8 ± 0.8	0.4 ± 0.5
Lower	2.8 ± 0.8[cd]	1.6 ± 0.4[c]	1.3 ± 0.5[d]

a – To analyze frequency of stained cells in relation to crypt topology, each depth compartment was semi-quantitatively assessed as a function of zone. A sample of 10 well stained cases were utilized, using a +1 to +3 scale (see Methods).
b – P-proximate to tumor; M- middle zone, proximate portion; D-distant zone; c – P/L vs. M/L, Mann-Whitney U-test, 1-tailed, non-significant, p>.1; d – P/L vs. D/L, p<.01

remote portion of this zone exhibited a gradual transition to the distant zone, **D**. The latter was characterized by mucosa of normal thickness, with only an isolated CCS. In 27% of cases, no Zone D was noted, in some instances even after 250–300 crypts from the tumor's edge. Only zone P exhibited an occasional crypt with mucin depletion and nuclear atypia consistent with mild dysplasia *(Table 1).*

Adenomatous polyps – Only two centrifugal zones were present (*Table 1*). The abnormal proximate zone – which had an intact muscularis mucosa – featured non-elongated CCSs in all specimens. The abnormal zone usually consisted of less than 25 crypts, as compared to carcinomas. In 45%, the adjacent mucosa was flattened and somewhat atrophic.

Aberrant crypt foci – The periphery of these small (<1.0 cm), slightly elevated mucosal lesions was characterized by two patterns (*Table 1*): CCS-rich mucosa (66%), and relatively normal morphology (60%). However, ACF differed from polyps because the majority of lesions (73%) exhibited only one of these morphological patterns. In this monophasic group 88% were non-dysplastic.

Flat adenomas and early carcinoma – These include 3 polyps with focally invasive (early) carcinoma and 5 flat adenomas; the latter exhibited moderate/severe dysplasia to focal (early) invasion. The malignant phases of these lesions did not extend to the tumor border. They are grouped together because of similar effects on the edge mucosa. Despite structural similarities to benign adenomatous polyps and ACF, respectively, the zonal changes were more similar to carcinomas then the latter lesions. In particular, the proximate zone mucosa was thickened with substantial crypt elongation in 88% of this group, similar to changes in zone M of carcinomas.

Immunocytochemical findings

Mild alterations in nuclear Ki67 expression occurred within crypts (vertical dimension) and between crypts (horizontal dimension). Only a rare crypt cell in Zone M expressed nuclear p53, so this marker was not further analyzed.

To capture these trends, two independent analyses of patterned Ki67 immunoreactivity were undertaken, as detailed in Methods. In the assessment of *expanded topology* each zone/case was scored for presence or absence of this change *(Table 2).* Since qualitatively, there was an impression that a preferential increase in *frequency* of stained cells was occurring in zone P, 10 cases were randomly selected for a second type of analysis. For that, frequency of stained crypt cells was semi-quantitatively analyzed in relation to each depth compartment as a function of zone *(Table 3).*

In zones P and M bordering carcinomas, mild to moderate expansion of the topological distribution of stained cells – towards the surface – occurred in almost all cases (*Table 2*). Zone M was divided into a proximate and distant part for this analysis.

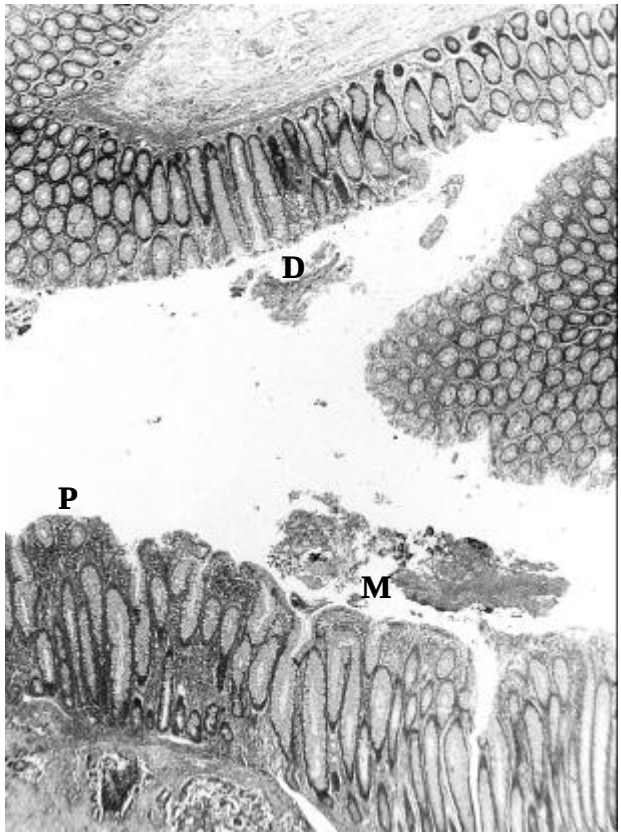

Figure 1. Panoramic view of the zonal organization at the edge of a carcinoma. Individual features of the zones are seen at higher magnification in other photographs. The proximate zone (P), is seen at the bottom left (cf. Figure 2,3). Area in bottom right, middle zone (M), shows crypt lengthening, increased complexity and density of crypts with connected crypt structures (cf. Figure 4,5). Transition between middle and distant zones, D, is seen in top strip, where the mucosa is of normal thickness and several straight crypts can be observed adjacent to "D". H&E, x28.

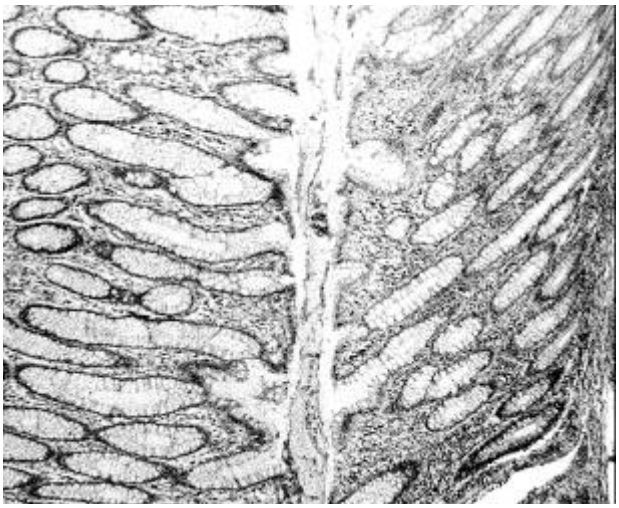

Figure 2. Proximate and middle zones to carcinoma. The middle zone, left, is characterized by markedly increased complexity and density of crypt architecture with connected crypt structures. The proximate zone, right contains fewer crypt profiles, which are elongated, and tortuously bent as evidenced by the series of linear, disconnected crypt profiles. H&E, x33.

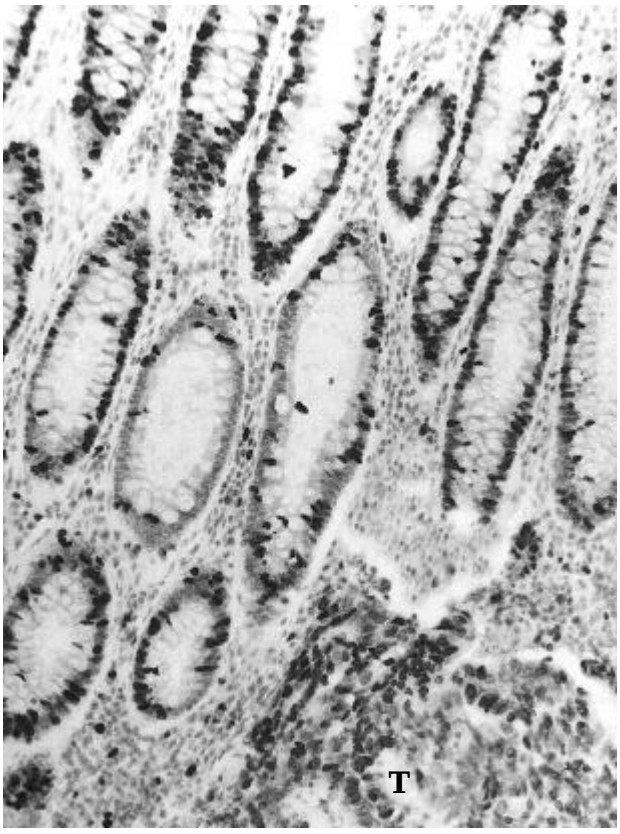

Figure 3. Proximate zone to carcinoma, stained for nuclear Ki67. An increase in immunoreactive nuclei is seen which is restricted to the basal and middle depth compartments. Tumor (T) is at lower right. Mayer's hematoxylin, x160.

Staining in the proximate zone of carcinoma was significantly more frequent than in the distant zone (Chi-square, $p<0.005$). No significant difference in expression was seen between zone P and M for Ki67. The semi-quantitative analysis of frequency as a function of depth compartment showed statistically comparable results *(Table 3).* However, there was a non-significant trend for zone P to exhibit increased frequency of stained cells and expanded topology in both modes of analysis.

For comparison we also examined 5 cases of adenoma peripheries (not shown in *Table 2).* Just as the histological changes were milder than that in carcinoma, so too was the extent of immunocytochemical expression. In 60% of proximate zones, Ki67 was mildly upregulated (vs. 94% in carcinoma).

Discussion

In this paper we document a consistent pattern of spatially ordered changes among crypts at the edge of colorectal carcinomas. This finding has two main implications, namely, pathogenesis and relationship to early neoplasia.

Previous investigations of immunocytochemical and histological changes in mucosa bordering a carcinoma, allow for a range of interpretations. Increased expression of proliferative cell markers and cytological atypia would be consistent with independent growth potential indicative of early neoplastic events.[3-8] The latter could reflect oncogenic mutations in bordering crypts.[9] Alternatively, these changes may merely represent regulated, growth responses to the progressively advancing tumor.[10-13]

One of the differences between malignant tumors, even of the same type, is their rate of growth into surrounding tissues.[20] Nevertheless, as the edge is remodeled by tumor invasion, zonal spatial relationships persist, as seen in almost all tumors in our series. There are several possibilities for maintaining this patterning, suggested by our data. In general terms, our findings can be accounted for by the concurrent activity of three pathogenetic processes involving crypts: trophic growth effects, destructive effects, and impaired crypt duplication manifested by complexes of connected crypts, *connected crypt structures* (CCSs).[16]

In carcinomas, crypts in the two adjacent zones always exhibited crypt elongation, associated with substantial mucosal thickening. The effect was not seen in borders of adenomatous polyps or ACF. Ki67 expression was also mildly augmented in the same territory. Consequently, this phenomenon could reflect a trophic influence of the carcinoma.[13] Although we are not aware of a specific trophic factor with this effect, crypt epithelial maturation is normally under humoral control,[25] and adenocarcinomas are well recognized as aberrant sources of growth factors for epithelial cells.[13] The finding of mucosal thickening with crypt elongation among early carcinomas and flat adeno-

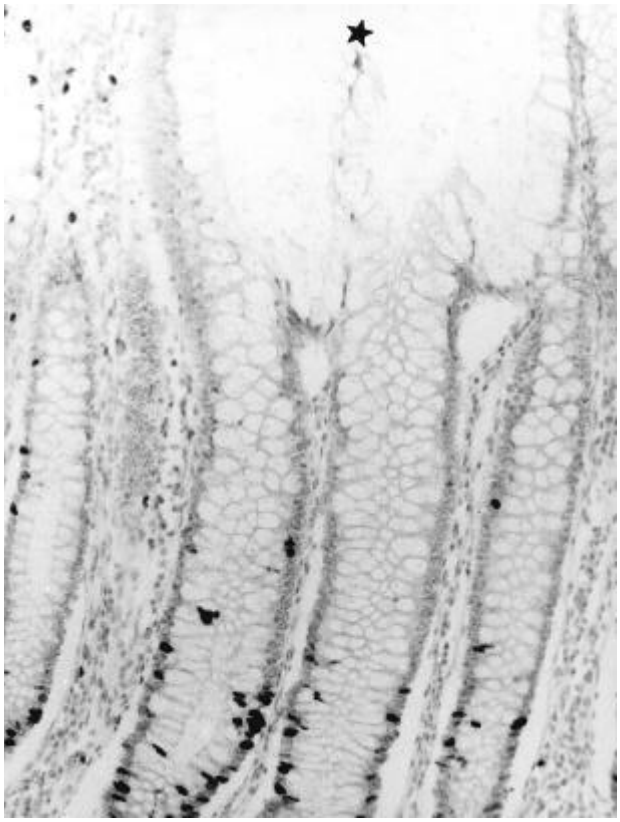

Figure 4. Normal expression of nuclear Ki67 in basal compartment of crypts from transition area between middle and distant zones. This nearly full thickness example shows a connected crypt structure, formed from crypts incompletely fissioned in the upper compartment. The star marks a shared wall between two daughter crypts, where their lumens enter a common cavity; the latter opens onto the mucosal surface at top of picture. The attachment points between daughter crypt are non-reactive. Mayer's hematoxylin, x154.

mas would also be consistent with a humoral trophic phenomenon. The latter were structurally quite similar to their more indolent counterparts, viz. adenomatous polyps and aberrant crypt foci, which lacked trophic manifestations.

A role for impaired crypt replicative cycling in the formation of CCSs is based on two findings. First, Ki67 expression did not occur at branch points in the superficial half of the mucosa, i.e. above the normal proliferative zone.[22] This suggests that CCSs are not a true proliferative budding process. On the other hand, crypts normally duplicate in a basal-to superficial splitting of a parent crypt into two daughter crypts,[26,27] which is a process distinct from epithelial renewal.[14] In diseases of the colon,[10,28] the crypts may remain attached, apparently as a result of failure of crypt replicative fissioning.[16,26] No known oncogenic mutation has been associated with similar changes.[29] Our finding of CCSs at the border of non-malignant lesions such as adenomatous polyps, aberrant crypt foci and even control mucosa, further supports the reactive pathogenesis of these changes.

Another likely pathogenetic factor is the destructive effects attributable to the carcinoma, most keenly manifest in the immediately adjacent proximate crypts. We found a lower crypt density there, with fewer connected crypts, but Ki67 expression statistically comparable to that in the Middle zone. Since such a picture is only seen in the region between the tumor and the middle zone, we surmise that invasion of the middle zone at its boundary with the tumor produces an evanescent domain that we recognize histologically as the Proximate zone. The simplification of crypt architecture in zone P compared to zone M is therefore attributed to loss of crypt elements. Since dysplasia was never observed in the middle zone, this cytological alteration probably indicates another cellular reaction to the destructive intrusion of the neighboring malignancy.

On the other hand, the patterning at the edge is unlikely to be due to major oncogenic mutational events.[9,16] Since such occurrences are rare,[19] we would anticipate a random distribution of alterations on this basis without consistent patterning between cases. Instead, the statistically signifi-

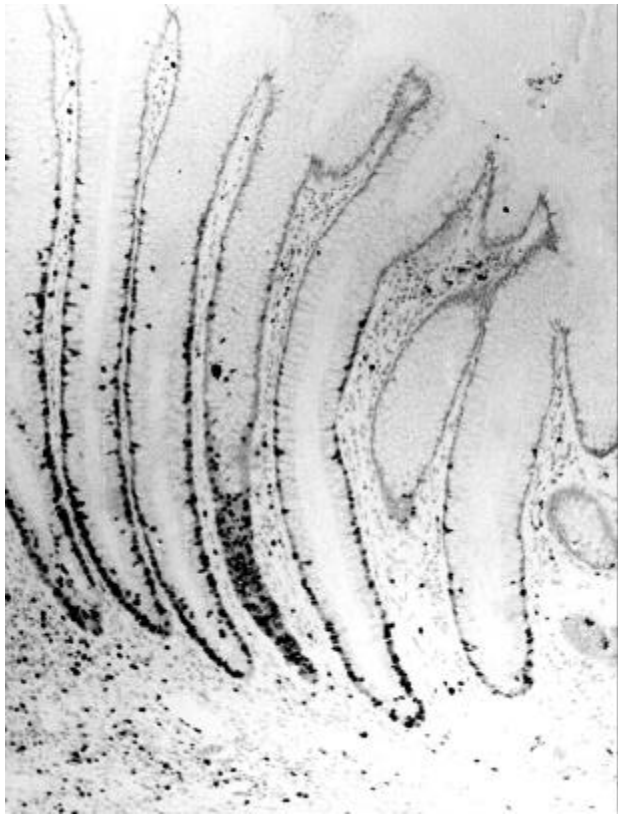

Figure 5. Middle zone, near carcinoma (Mp) stained for nuclear Ki67. Mildly expanded topology of immunoreactive nuclei, extending throughout basal and mid depth compartments, reaches the superficial compartment of these elongated crypts. Compare Figure 4. Mayer's hematoxylin, x77.

cant consistency of these findings across cases suggests they are not random occurrences, nor do they represent a simple proximal-distal gradient of change. Although *in situ* genetic analysis would be necessary to conclusively examine this issue, expression of p53 at the border showed no indication of mutated phenotype in any case, and Ki67 was always topologically restricted. This is in contrast to carcinomas where Ki67 looses its topological localization,[20] and p53 is commonly expressed at high levels.[21]

We conclude that the typical pattern of morphological and molecular changes at the tumor's edge most likely represent a comples physiological response to the adjacent malignancy, rather than an independent process of neoplastic transformation. These spatially organized reaction patters appear to reflect an interplay betwen regulatedgrowth and destructive processes in response to adrancing carcinoma. Regardless of pathogenesis, the features of carcinomas' edge mucosa are so distinctive that they may be useful diagnostically. A biopsy with these changes alone should raise the suspicion that a nearby carcinoma was not sampled.

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