The Mode of Growth and Compartmentalization of Neoplastic Glands During Experimental Colon Carcinogenesis

WILLIAM W. L. CHANG, MD, PhD

From the Department of Pathology, West Virginia University School of Medicine and University Hospital, Morgantown, West Virginia

During the growth of 1,2-dimethylhydrazine (DMH)-induced colon neoplasms in mice from microscopic ones at 9 weeks to macroscopic, invasive ones at 25–26 weeks after the initiation of DMH treatments, the neoplastic glands became increasingly but variably elongated and tortuous, with epithelial evaginations and/or invaginations. For assessment of the mode of growth and genesis of heterogeneity of neoplasms, colon neoplasms induced by two different cumulative doses of DMH were compared at 25–26 weeks after the initial DMH injection. At this time they invaded the colonic wall similarly in depth. However, neoplasms that developed in mice given a higher cumulative dose of DMH had a more homogeneous cell population, a higher proliferative activity, and more apoptotic bodies than those with a lower dose. By 73 hours after multiple tritiated thymidine injections, most neoplastic cells became labeled. There were numerous foci of unlabeled cells seen among, or alternating with, areas of labeled cells. Epithelial evaginations into the glandular lumen consisted of proliferating cells and/or differentiated cells; whereas invaginations into the lamina propria contained only proliferating cells. These findings suggest a compartmentalization of neoplastic glands into multiple neoplastic clonogenic units during growth, from which cellular heterogeneity and architectural complexities of neoplastic glands develop. (Am J Pathol 1986, 124: 420–426)

THE CELLULAR HETEROGENEITY of neoplasms, including colonic cancer, has been known histologically since the last century. Several phenotypic and karyotypic features showing the heterogeneity of tumor cell lines derived from human colon carcinomas have also been demonstrated.[1,2] However, it still remains to be elucidated when and how such cellular heterogeneity develops in colonic carcinomas, particularly in relation to the growth and progression of the neoplasms *in vivo* on morphologic grounds, because cancer is usually considered to be a clonal disease.[3,4]

In this investigation, colonic epithelial neoplasms were induced by weekly injections of symmetric 1,2-dimethylhydrazine (DMH) in mice,[5] and the modes of neoplastic growth and genesis of cellular heterogeneity in the neoplasms were studied with the use of autoradiographs prepared from Epon-embedded sections of colonic neoplasms of mice given single or multiple injections of tritiated thymidine (TdR). Proliferating and nonproliferating neoplastic cells were identified by TdR labeling in the autoradiographs and correlated with their cytologic features, as established in our previous studies.[5–8] Their distribution was analyzed in relation to the changing patterns of growing neoplastic glands, particularly the glandular elongation and tortuosity, epithelial evaginations and invaginations, as well as daughter gland formation. Meanwhile, the relative frequency of TdR-labeled and unlabeled neoplastic cells, apoptotic bodies (*vide infra*), and desquamated cells was quantitated in colon neoplasms induced in mice given different cumulative doses of the carcinogen.

The purpose of this investigation was 1) to elucidate the mode of development of cellular heterogeneity and glandular complexity in colonic neoplasms with growth;

Accepted for publication April 23, 1986.

Address reprint requests to William W. L. Chang, MD, PhD, Department of Pathology, West Virginia University, Medical Center, Morgantown, WV 26506.

2) to study the effect of the different cumulative doses of the carcinogen on the biological behavior and cellular heterogeneity of colon neoplasms; and 3) to investigate the role of cell loss and apoptosis on cellular proliferation and differentiation of colonic neoplasms. The preliminary results of this investigation were presented previously.[9]

Materials and Methods

Young adult female CF-1 mice 12–14 weeks of age (Charles River Breeding Laboratories, North Wilmington, Mass) were used. They were maintained on Purina Laboratory Chow and water *ad libitum* in an air-conditioned room with a 12-hour light cycle.

For induction of colonic neoplasms, the mice were given weekly subcutaneous injections of 20 mg DMH-2HCl (Aldrich Chemical Co., Milwaukee, Wis)/kg body weight. The chemical was dissolved in 1.5 mM EDTA solution, and its pH was adjusted to 6.5 with sodium hydroxide before use.

To assess the mode of growth of colon neoplasms with time, we gave 4 mice DMH subcutaneously once a week for 9 weeks, 4 mice for 11 weeks, and 4 mice for 18 weeks, and they were killed 1 week after the last DMH injection; ie, the time of sacrifice was 9, 11, and 18 weeks after the initiation of DMH treatments. Forty minutes before killing, these mice received 1 μCi/g body weight of TdR (specific activity, 11 or 56 Ci/mmol).

In the main experiment, which was designed to assess the effect of different cumulative doses of DMH on the biologic behavior of chemically induced colonic neoplasms, 35 mice were divided into two groups. Seventeen mice received subcutaneous injections of DMH once a week for 12 weeks (Group I), and 18 mice, for 22 weeks (Group II). The mice were examined weekly for their body weight and abnormal signs. One of 17 mice in Group I and 4 of 18 mice in Group II died before completion of the experiment. Twenty-five weeks after the commencement of DMH treatments, 4 mice in Group I and 3 mice in Group II were killed 1 hour after a single injection of 1 μCi/g body weight of TdR (specific activity, 11 Ci/mmol), and again 4 mice in Group I and 3 mice in Group II were sacrificed at 3 hours after a single injection of the same dose of TdR. Macroscopic, often invasive colon carcinomas present in these animals were compared with those mostly microscopic neoplasms in mice that were killed at the earlier times, ie, at 9, 11, and 18 weeks. Meanwhile, the remaining 8 mice in each group were administered multiple injections of 0.5 μCi/g body weight of TdR every 8 hours, and they were killed in a group of 4 mice in both Groups I and II at 25 and 73 hours, respectively, after the initial or 1 hour after the last TdR injection. By giving multiple injections of TdR at an interval of about or less than the DNA synthesis time, we hoped to label all the neoplastic cells that would enter the proliferative cycle during these experimental periods, and the proliferative activity and the turnover rate of the neoplastic cells could be estimated.

A necropsy excluding the head was performed at the time of sacrifice, and the gross appearance of the colon and other visceral organs was recorded. A segment of the distal colon between 1 and 4 cm from the anus, including neoplasms, if any, was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). During fixation, the colonic segment was cross-sectioned to obtain four to six representative ringlike structures, and all the grossly identifiable colonic tumors, if any, were dissected out separately. They were dehydrated through graded concentrations of acetone and embedded in Epon. One-micron-thick, semiserial Epon sections were stained with periodic acid-Schiff (PAS) reaction and iron-hematoxylin[6] and then processed for autoradiography[10] with the use of NTB-2 emulsion (Eastman Kodak Co., Rochester, NY). The exposure time was 4 weeks. The background silver grains were negligible in the autoradiographs thus prepared. In the main experiment, 4 mice from each group that received TdR every 8 hours and killed at 73 hours after the initial TdR injection were employed for cell counting. In each mouse, two neoplasms were analyzed; and in each tumor, 1800–3500 nuclei were counted and analyzed. The quantitative data on cell counts were analyzed statistically with the Student *t* test, because the data obtained roughly followed a normal distribution.

Results

Chemically Induced Colonic Neoplasms

Although not apparent macroscopically, dysplastic crypts and small neoplastic lesions were observed sporadically in the colonic mucosa under light microscope in all 4 mice killed at 9 weeks after the initiation of DMH treatments. These neoplastic lesions, which were very small and represented early lesions, were usually located in the upper part of the mucosa, as documented previously.[5,7,8] Possible modes of formation of dysplastic crypts and of progression from dysplastic crypts to small neoplastic lesions were also discussed previously.[5,7,8] By 11 weeks, the neoplastic lesions appeared to be more numerous and enlarged in all 4 mice, as compared with those seen at 9 weeks, but their leading downward edge was well within the upper two-thirds of the mucosa. By 18 weeks, there was a further increase in the size of the neoplasms. Although these neoplasms were often detected macroscopically, they were still

Table 1—Leading Edge of Most Advanced Colon Neoplasms

No. of DMH injections	Total cumulative dose of DMH (mg/kg)	Weeks from start of treatment to killing	Incidence of tumors*	Location of leading edge of most advanced neoplasms			
				Whole mucosa	Submucosa	Muscularis propria	Serosa
12	240	25–26	16/16	6	7	2	1
22	440	25–26	14/14	4	8	2	0

* The incidence is expressed by the number of tumor-bearing mice per total number of mice studied.

within the mucosa and did not penetrate the muscularis mucosae in the 4 mice studied.

At 25–26 weeks after the initiation of DMH injections, all the surviving mice, whether they received 12 or 22 weekly injections of DMH, developed multiple neoplasms in the distal colon. Because in most animals, the distal 4–5 cm of the colon was carpeted with numerous tumors and it was very difficult to count the number of tumors with accuracy, the quantitation of neoplasms in the colon was not performed. When the depth of invasion of the most advanced neoplasms in the colonic wall was analyzed in each animal, there was no significant difference between the two groups of mice that received 12 and 22 weekly injections of DMH (Table 1).

Cytokinetic Aspects

In the chemically induced colon neoplasms, many, but variable, numbers of desquamated cells and their debris were present in the lumen of the neoplastic glands. Scattered neoplastic cells contained in their cytoplasm phagolysosomes with degenerating cellular or nuclear debris, known as apoptotic bodies.[11] Quantitatively, more apoptotic bodies were found in neoplasms induced in mice with 22 weekly injections of DMH than in those with 12 weekly injections. However, the relative frequency of desquamated nuclei within the neoplastic glands did not appear to differ significantly between the two groups of mice (Table 2), although these desquamated cells exhibited regional variations and tended to be underestimated.

After TdR injections every 8 hours for 73 hours, significantly more labeled apoptotic nuclei and desquamated nuclei were found in colonic neoplasms in mice that received 22 weekly injections of DMH than those given 12 injections. Moreover, the TdR labeling of neoplastic cells was also significantly higher in the former (93.8%) than in the latter (79.6%) (Table 3). Hence, the turnover rate of neoplastic cells in the former was estimated to be 1.28% cells per hour, whereas that in the latter was 1.09% cells per hour. In other words, the turnover time of the neoplastic cells in the former was 77.8 hours, and that in the latter was 91.7 hours. The turnover time of both groups of neoplasms was much shorter than that of the colonic epithelium of the normal mouse.[12]

Histopathologic Aspects

Employing semiserial sectioning of multiple sections of neoplasms, we reconstructed the growth processes of colonic neoplasms based on the different times of killing mice after the commencement of DMH treatments and the changes in the size and histopathologic features of neoplasms with time, although we were aware of limitations of studying fixed tissue sections.

Early small neoplastic lesions that were observed by 9 weeks in the upper portion of the colonic mucosa consisted of relatively simple glandular structures lined with a homogeneous population of atypical, proliferating cells.[5,7,8] As small microscopic neoplasms became enlarged and progressed to macroscopically visible ones from 9 weeks to 25–26 weeks, there were variable degrees of changes in the neoplastic glandular configuration: elongation and tortuosity of neoplastic glands, evaginations and/or invaginations of their lining epithelium, and division of neoplastic glands, as a result of inces-

Table 2—Incidence of Apoptotic Bodies and Desquamated Nuclei in Chemically Induced Colon Neoplasms

No. of DMH injections	No. of tumors analyzed	Percentage of		
		Neoplastic nuclei	Apoptotic nuclei	Desquamated nuclei
12	8	94.5 ± 1.8*	1.6 ± 0.5	3.9 ± 1.5
22	8	94.5 ± 1.8	2.8 ± 0.7†	2.7 ± 1.5

* Mean ± SD.
† $P < 0.01$

Table 3—Frequency of Labeling of Neoplastic, Apoptotic, and Desquamated Nuclei at 73 Hours After Multiple Injections of Tritiated Thymidine

No. of DMH injections	No. of tumors analyzed	Percentage of labeled nuclei		
		Neoplastic nuclei	Apoptotic nuclei	Desquamated nuclei
12	8	79.6 ± 7.0*	60.5 ± 11.4	48.8 ± 13.2
22	8	93.8 ± 4.0†	78.2 ± 7.8†	78.8 ± 11.3†

* Mean ± SD.
† $P < 0.01$.

sant proliferation and loss of neoplastic cells and the status of the basement membrane of the glands in the given areas.[5] Concomitant with neoplastic growth, some neoplasms maintained a relatively homogeneous cell population, and others became variably heterogeneous in their constituent cell population. In the latter, variable degrees of differentiation of neoplastic cells occurred in relation to the changing glandular configuration. The differentiation of neoplastic cells could be judged, on the basis of the differentiation of epithelial cells in the normal colonic crypt,[6] cytologically by the changes from cells with a basophilic cytoplasm and a darkly stained nucleus to those with a pale cytoplasm and a lightly stained, finely granular nucleus, and autoradiographically by the lack of labeling with TdR.

At 25–26 weeks after the initial DMH treatment, the mice that received 22 weekly injections of DMH had relatively more neoplasms that were composed of atypical glands lined with a morphologically homogeneous population of neoplastic cells, nearly all of which were labeled with TdR by 73 hours (Figure 1). In mice that received 12 weekly injections of DMH and were killed at the same time, considerably more neoplasms displayed varying degrees of cellular differentiation and revealed scattered unlabeled cells after TdR injections. Although variable within and among neoplasms, they were present singly or in clusters among or alternating with areas of proliferating cells in the neoplastic glands (Figures 2–4). Their distribution was such that the neoplastic glandular epithelium was seemingly partitioned into multiple small units of varied sizes with unlabeled cells forming the boundaries. Such a partition or compartmentalization tended to be more apparent in tortuous or irregular glands (Figure 2).

An early evagination of neoplastic glandular epithelium into the glandular lumen often consisted of TdR-labeled cells (Figure 2); but as the growth continued, the evaginated epithelium was frequently composed of both proliferating and nonproliferating cells (Figure 4). In certain glands, a cribriform pattern was observed (Figure 5), and both proliferating and nonproliferating cells were present. Nonproliferating cells manifested cytologic differentiation toward columnar absorptive cells and sometimes goblet mucous cells (Figure 5). Whenever an invagination of the neoplastic glandular epithelium into the underlying lamina propria occurred, the constituent neoplastic cells were labeled with TdR (Figures 1, 3, and 4). When the invaginated area grew to form a daughter glandular structure from the neoplastic gland, only proliferating cells were encountered. However, the growth of the daughter glandular structure might later be associated with differentiation of some of its constituent cells. No definite pattern of distribution of apoptotic bodies was observed, but they

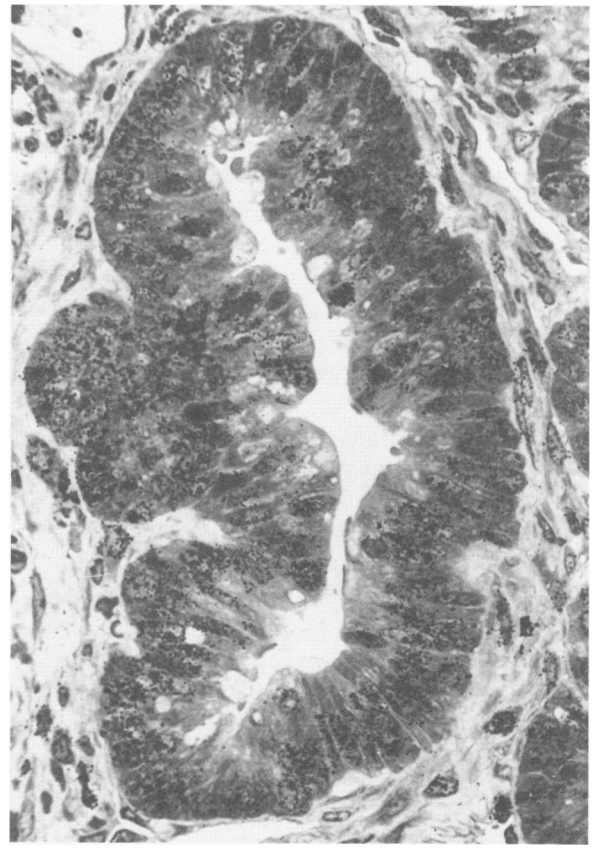
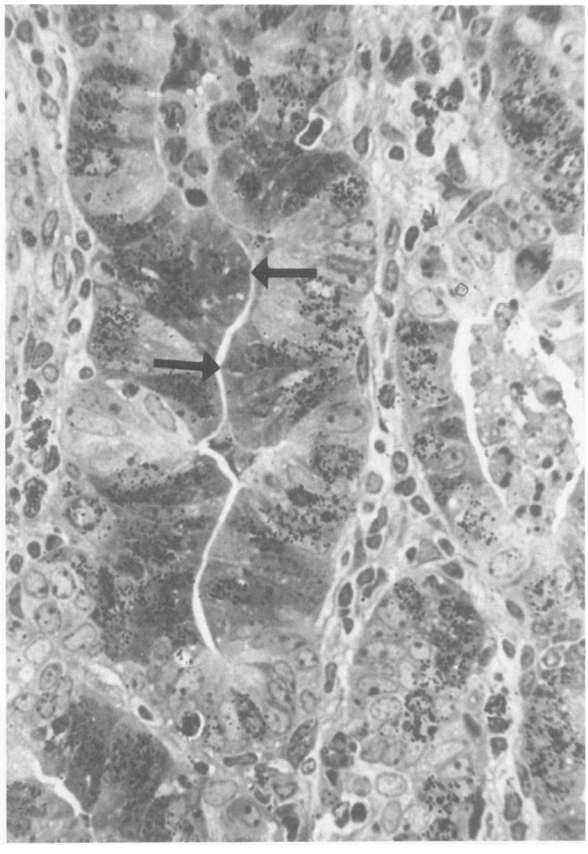
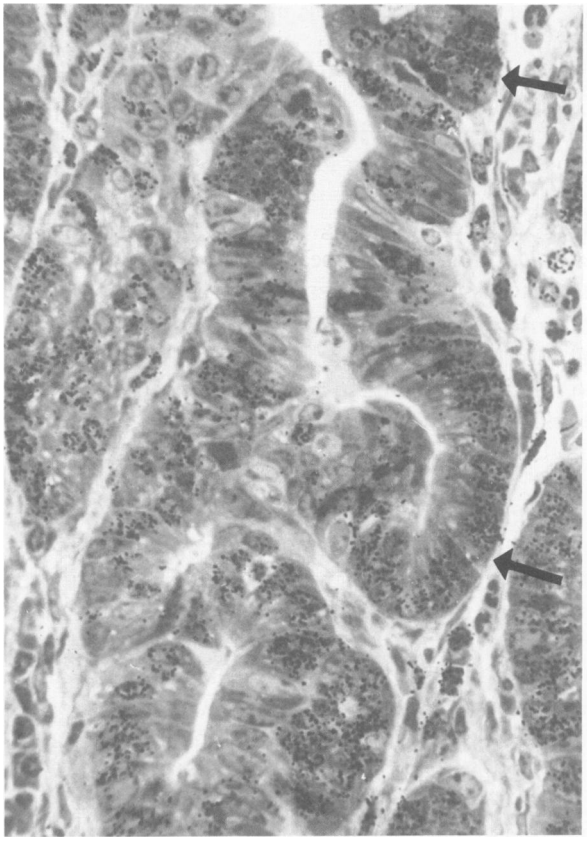


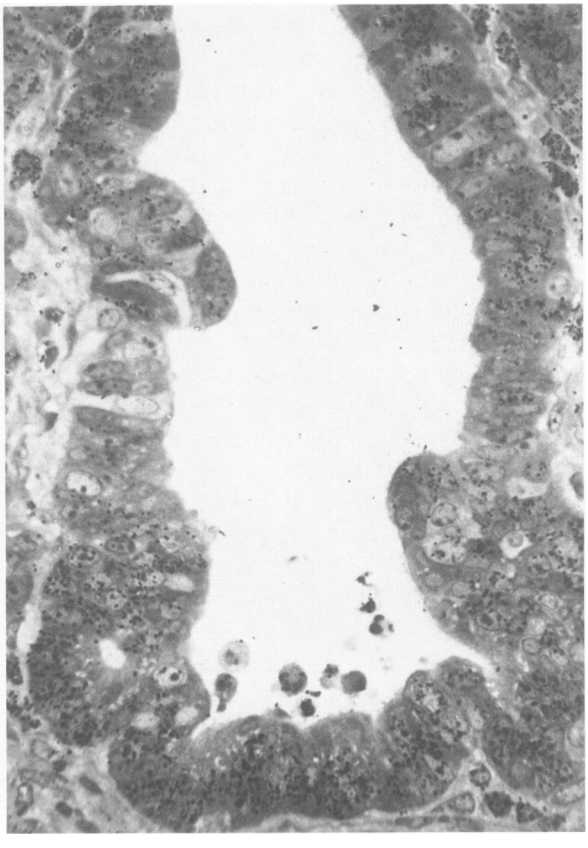
Figure 1—Autoradiograph at 73 hours after multiple injections of TdR, exhibiting neoplastic glands from a growing colon neoplasm. Almost all the neoplastic cells were labeled with TdR. Note a few foci of epithelial invagination into the lamina propria. This and the following photomicrographs were taken from 1-μ-thick Epon-embedded sections stained with PAS and iron hematoxylin. (×640)



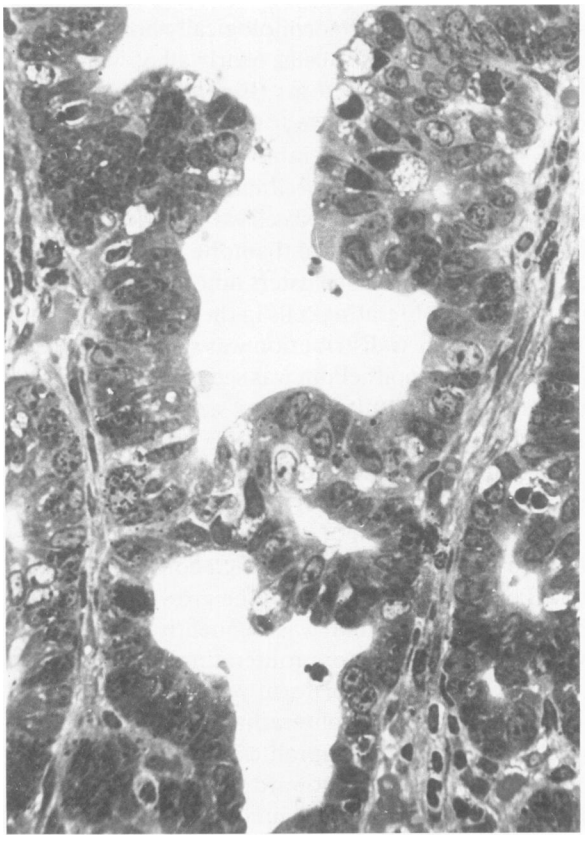
2



3



4



5

tended to be present within proliferating cells. Many neoplastic glands contained varying numbers of desquamated cells or nuclei in their lumens.

The neoplastic glands invading the submucosa or muscularis externa were often composed of proliferating and nonproliferating cells, even their leading downward edges. Some of the nonproliferating cells exhibited varying degrees of cytologic differentiation. Although the distribution of TdR-labeled and unlabeled cells suggested the compartmentalization of the invading glands, the manner of such partition seemed to be irregular. Apoptotic bodies and desquamated cells or nuclei were also present in varying numbers in the invading neoplastic glands.

Discussion

The production of chemically induced colonic neoplasms depends on the cumulative dose of the carcinogen given to animals.[13,14] The current study indicates that the depth of invasion of DMH-induced neoplasms in the colonic wall did not rely significantly on the cumulative dose of the carcinogen; rather, it appeared to be a factor of time between the initial treatment and sacrifice. However, neoplasms that developed in animals given a higher cumulative dose of DMH tended to have a more homogeneous cell population and had more cells in the proliferative cycle than those produced in animals given a lower dose, although some variations existed in neoplasms within and among animals. A higher turnover rate of neoplastic cell in the former was also evident from an increase in the number of apoptotic bodies and also of TdR-labeled apoptotic bodies and desquamated nuclei. Our findings support the hypothesis that neoplasms produced by a higher cumulative dose of carcinogen have a higher degree of alteration in the DNA and other macromolecules in their constituent cells, and thus they exhibit an increased rate of cell proliferation and turnover and restricted cell differentiation.

Morphologically, a first step for the development of the colonic neoplasm is a repopulation of a colonic crypt by atypical cells (dysplastic crypt), which leads to the formation of a small neoplastic lesion in the upper part of the crypt.[5,7,8] The latter appears to represent a neoplastic clonogenic focus, from which a tumor develops, consistent with the clonal origin of the neoplasm.[3,4] Because the small neoplasm is composed of a homogeneous population of atypical, proliferating cells, those cells appear to be consistent with neoplastic stem cells.[15]

The mode of growth from such a neoplastic clonogenic focus to a full-fledged tumor varies from tumor to tumor, probably because of different degrees of concerted but sequential interaction of genetic and epigenetic regulatory mechanisms altered by the repeated administration of the carcinogen. In the colonic epithelial neoplasms, this mode is histopathologically manifested by elongation and tortuosity of neoplastic glands, evaginations and invaginations of their lining epithelium, and division of neoplastic glands, thereby increasing the size and the number of the neoplastic glands,[5] in association with varying degrees of cell differentiation. These changes of neoplastic glands appear to be best explained by the concept of compartmentalization of the glands into multiple small clonogenic units with growth. Although further studies are required, particularly in sequential and three-dimensional terms, our studies favor the contention that the epithelium of the growing neoplastic glands is successively partitioned into multiple small structural units, each of which can be considered as a neoplastic clonogenic unit. Such units can be detected when nonproliferating cells constitute their boundaries. However, when the neoplastic glandular epithelium is lined with a homogeneous population of atypical, proliferating cells, such units can hardly be appreciated unless further alterations of the configuration of the neoplastic glands take place.

It appears that epithelial evaginations and invaginations of the neoplastic glands are functions of neoplastic clonogenic units. They are initiated by clusters of proliferating cells within the units, and the growth of epithelial evaginations, but not of invaginations, is often associated with differentiation of the constituent cells. The process of epithelial evagination is reminiscent of the process of dichotomy of the crypt[5,8] or of villous formation during development of the gut epithelium. Growth of epithelial evaginations would lead to complete or incomplete division of the neoplastic glands as in the process of dichotomy of the crypt, if connective tissue growth accompanies epithelial evaginations. Concomitant formation and growth of epithelial evaginations from several clonogenic units within the neo-

Figure 2—Autoradiograph at 25 hours after TdR injection. A slightly tortuous neoplastic gland contained clusters of unlabeled cells among or between groups of labeled cells. Early evaginations into the glandular lumen (*arrow*) were associated with labeled cells. (×640) **Figure 3**—Autoradiograph at 25 hours after TdR injection. In the tortuous neoplastic gland, epithelial invaginations into the lamina propria (*arrow*) were associated with labeled cells. (×640) **Figure 4**—Autoradiograph at 73 hours after TdR injection. In the dilated neoplastic gland, a few unlabeled cells were present in clusters among labeled cells. Epithelial evaginations into the glandular lumen were associated with labeled and unlabeled cells. (×640) **Figure 5**—Autoradiograph at 25 hours after TdR injection. The dilated neoplastic gland exhibited epithelial evaginations with an early cribriform pattern formation in one area. These evaginated areas were composed of labeled and unlabeled cells. A few goblet mucous cells were present. (×640)

plastic gland may result in the formation of the cribriform pattern of neoplastic glands. On the other hand, epithelial invagination of the neoplastic glands into the lamina propria is probably associated with changes in the basement membrane, often seen in malignant glands. The mode of epithelial invagination mimics the formation of crypts in the gut epithelium during embryonal development. Successive changes of neoplastic glands by these processes probably lead to more complex glandular structures and heterogeneity of the neoplasms.

Invading neoplastic glands in the colonic wall are often lined somewhat haphazardly with both proliferating and nonproliferating cells, also suggesting the compartmentalization of the glands into multiple clonogenic units. It should be noted that the invasive behavior of neoplastic glands is not a result of increased cell proliferation,[5,8] but, rather, related to elaboration of special proteins or enzymes by neoplastic cells.[16] These invading glands are often surrounded by concentric layers of collagen and fibroblasts, manifesting desmoplasia.[5] The circumstantial evidence suggests that the invading glands have been pinched off from the mother glands after invagination and infiltrate the stroma independently.

Hence it appears that proliferation and differentiation of neoplastic cells occur constantly throughout the process of growth and progression of colonic neoplasms. As a consequence, neoplastic glands appear to be successively compartmentalized into multiple clonogenic units, from which epithelial evaginations and invaginations may develop, leading to more complex glandular structures and heterogeneity of the neoplasm. It is possible that each clonogenic unit is different, and certain subpopulations of neoplastic cells may originate from some clonogenic units to initiate different biologic behavior. The above concept, if proven to be correct, may have great implications in understanding the biologic behavior of neoplasms and also in therapeutic strategies.

References

1. Dexter DL, Spermulli EN, Fligiel Z, Barbosa JA, Vogel R, VanVorhees A, Calabresi P: Heterogeneity of cancer cells from a single human colon carcinoma. Am J Med 1981, 71:949–956
2. Brittain MG, Levine AE, Chakrabarty S, Yeoman LC, Wilson JKV, Long B: Heterogeneity of human colon carcinoma. Cancer Metastasis Rev 1984, 3:177–191
3. Nowell PC: The clonal evolution of tumor cell populations. Science 1976, 194:23–28
4. Fialkow PJ: Clonal origin of human tumors. Biochim Biophys Acta 1976, 458:283–321
5. Chang WWL: Histogenesis of colon cancer in experimental animals. Scand J Gastroenterol 1984, 19(Suppl):27–43
6. Chang WWL, Leblond CP: Renewal of the epithelium in the descending colon of the mouse: I. Presence of three cell populations: Vacuolated-columnar, mucous and argentaffin. Am J Anat 1971, 131:73–100
7. Chang WWL: Histogenesis of symmetrical 1,2-dimethylhydrazine-induced neoplasms of the colon in the mouse. J Nat Cancer Inst 1978, 60:1405–1418
8. Chang WWL: Morphological basis of multistep process in experimental colonic carcinogensis. Virchows Arch [Cell Pathol] 1982, 41:17–37
9. Chang WWL: Cell proliferation, differentiation and death (apoptosis) in experimental colonic carcinogenesis (Abstr). Fed Proc 1985, 44:415
10. Kopriwa BM, Leblond CP: Improvements in the coating technique for radioautography. J Histochem Cytochem 1962, 10:269–284
11. Kerr JFR, Wyllie AH, Currie AR: Apoptosis: A basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer 1972, 26:239–257
12. Chang WWL, Nadler NJ: Renewal of the epithelium in the descending colon of the mouse. IV. Cell population kinetics of vacuolated-columnar and mucous cells. Am J Anat 1976, 144:39–56
13. Druckrey H: Production of colonic carcinomas by 1,2-dialkylhydrazines and azoxyalkanes, Carcinoma of the Colon and Antecedent Epithelium. Edited by WJ Burdette. Springfield, Ill, Charles C Thomas, 1970, pp 267–279
14. Deschner EE, Long FC, Maskens AP: Relationship between dose, time and tumor yield in mouse dimethylhydrazine-induced colon tumorigenesis. Cancer Lett 1979, 8:23–28
15. Steel GG, Stephes TC: Stem cells in tumours, Stem Cells: Their Identification and Characterization. Edited by CS Potten. Edinburgh, Churchill Livingstone, 1983, pp 271–293
16. Liotta LA, Rao CN, Barsky SH: Tumor invasion and the extracellular matrix. Lab Invest 1983, 49:636–649