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Cell Proliferation in Colorectal Tumor Progression: An Immunohistochemical Approach to Intermediate Biomarkers

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Abstract Cell renewal in the large intestine mucosa is normally tied to a rigidly compartmentalized model. Immunohistochemical identification of cells in S phase through uptake of bromodeoxyuridine is the method of choice for detailed compartmental mapping of proliferation, while immunohistochemical detection of proliferation-associated antigens (Ki-67, PCNA, DNA polymerase α) provides information in advanced tumor cases. Mucosal hyperproliferation due to inflammation may be transient (self-limited colitis, Crohn's disease, acute radiation damage) or lasting (ulcerative colitis). Progressive shifting of the proliferation zone to the crypt surface (Stage II abnormality) is a late feature of irradiated rectal mucosa and subgroups of ulcerative colitis patients at high risk for cancer. Hyperproliferation and Stage II abnormality coexist in the mucosa of patients with colorectal neoplasia, but are mutually independent and correlated to different clinical and pathological features of the disease. These cytokinetic abnormalities are highly predictive markers of the adenoma-carcinoma sequence, but are not associated with *de novo* adenocarcinoma. Proliferation increases progressively in the subsequent steps of this sequence, except in early cancer.

Key words: cancer risk, cell proliferation, chemoprevention, colonic mucosal hyperproliferation, colorectal adenomas, colorectal cancer, immunohistochemistry, intermediate biomarker, intestinal mucosa

Proliferation is clearly a central feature of cell biology, since it ensures the anatomical and functional intactness of the tissues. It is the basic homeostatic regulator of the tissue trophism insofar as its increase and decrease result in hyperplasia and atrophy, respectively. Moreover, it is closely correlated with carcinogenesis. Marked changes in cell proliferation, in fact, occur in the early stages of carcinogenesis and persistence of hyperproliferation plays a decisive role in the production of a neoplasm [1]. Howard and Pelc's model [2] of tissue kinetics divides the cell pool into a proliferative compartment composed of cells actively engaged in the several phases of the cycle, and cells that are irreversibly or reversibly outside the cycle (non-cycling cells). In truth, however, cell renewal is a complex, organized system formed of populations and subpopulations differing kinetically and in their coordinated flows and chronological sequences. In situ exploration of this system permits the quantification of proliferation through numerical parameters, such as the labeling index (LI), that can readily be compared and processed. Above all, it allows identification of populations in particular moments or attitudes of proliferation and relation of these findings to their histological and cytological features. There is, in fact, a wide range of variety in the proliferation characteristics of different districts in the same tissue, so much so that proliferative zones and/or compartments may be located in histological microstructures with a rigid architectural or spatial organization. Compartmentalization of proliferation is readily detected in the epithelia of the gastrointestinal mucosa [3], as well as in other districts (bladder, cervix, etc.) [4,5]. Even tissues whose morphology displays little evidence of a strict

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architectural arrangement (the hemopoietic marrow is a possible example) present cell proliferation distribution gradients [6]. The qualification of biomarkers can thus be attached to stages of premorphological abnormalities of proliferation compartmentalization referable to different cancer risk levels according to various predictive values. Colorectal mucosa cell renewal is tied to a rigidly compartmentalized model. In Lieberkhun's crypt, the proliferative zone is restricted to the intermediate and deep portions, whereas the superficial third (the so-called functional compartment) is populated by cells involved in differentiation processes, but not in proliferation, migrating toward the surface in an orderly manner [3]. The LI drops to zero in this zone in keeping with the model of Carnie, Lamerton and Steel [7].

A REVIEW OF THE METHODS

As a first step, critical assessment must be undertaken of all the methods potentially employable in detailed compartmental mapping of proliferating cells in the crypt. The main features of intestinal cell proliferation were established by Lipkin [3] during the sixties through autoradiographic identification of cells in the S-phase by their uptake of tritiated thymidine, and until 1985 this subject was primarily investigated by means of autoradiography. Today several immunohistochemical methods are available for the evaluation of cell proliferation. Two have already been sufficiently tried and tested.

(1) Nuclear uptake of pyrimidine analogs of thymidine effectively labels S-phase cells. Bromodeoxyuridine (BrdU) and Iododeoxyuridine (IdU) are the halopyrimidines most frequently employed.

(2) Proliferation-associated antigens, i.e., endogenous antigens, are unequivocally expressed in one or several phases of the cell cycle.

BrdU Incorporation

BrdU is a thymidine analog with bromine in place of the methyl group in position 5 of the pyrimidine ring. It is readily taken up by DNA as an alternative to thymidine in the synthesis stage. Various, mostly monoclonal, antibodies recognize incorporated BrdU and label the newly synthesized DNA. S-phase cells are then identified with routine immunohistochemical methods [8]. Nuclear immunostaining is easily identified in the absence of a background in the form of both diffuse, uniform staining of the entire nuclear surface, and localized positivity confined to the edge of the nucleus presenting as a ring composed of variously coalescent granules. This phenomenon may be due to differences in the duration of BrdU uptake by DNA, some cells being in the early or middle S-phase, and others in the late S-phase. Exposure to BrdU can be conducted in vitro on microslices or by i.v. infusion. In vitro incubation presupposes the vitality and appropriate size of the tissue specimen, since BrdU diffusion and uptake are a function of the distance from the interface between the tissue and the medium [8], as well as the structural features of the tissue itself (desmoplastic reaction, mucinous inhibition of the matrix, etc.). The size and histology of endoscopic intestinal mucosa biopsy specimens are in any event such as to ensure uniform labeling over the whole surface of a histologic section. Immunohistochemical application of the BrdU/anti-BrdU MAb method is less time-consuming than autoradiography. Several papers have shown that both techniques are equally accurate in the detection of S-phase cells and the coefficient of correlation between their values is excellent [9]. Simultaneous immunocytochemical detection of BrdU uptake and spontaneously expressed differentiative antigens permits investigation of the kinetics of specific subpopulations. We have observed proliferative abnormalities in rectal mucosa neuroendocrine cells during ulcerative colitis by means of double immunostaining: in vitro labeling of the nucleus with BrdU, and cytoplasmic labeling of antigens correlated with neuroendocrine differentiation (Chromogranin A, Synaptophisin) [10]. In situ characterization of tissue proliferation can be completed by determining other parameters, such as S-phase duration, in addition to the LI (i.e., the fraction of cells in S-phase). The rate of progression into the cycle is basic for extrapolation of the duration value. It can be calculated by using the sequential uptake of two thymidine precursors

to determine the fraction of cells leaving the S-phase per unit of time. Monoclonal antibodies distinguishing different halopyrimidines are now available. Br-3 MAb only recognizes BrdU, while IU4 MAb recognizes both BrdU and IdU. Immunohistochemical detection of sequentially incorporated BrdU and IdU by immunostaining with alkaline phosphatase/anti-alkaline phosphatase, and with the avidin-biotin-peroxidase complex, ensures effective chromatic discrimination of different nuclear populations [11]. Modifications of S-phase duration have been described in large intestine tumors [12]. Measurement of this cytokinetic parameter is laborious, however, and its use as a biomarker is confined to small, selected groups.

Proliferation-Associated Antigens

Immunohistochemical detection of proliferation-associated antigens provides a different approach to the investigation of cell proliferation in situ. No exposure to or uptake of DNA precursors is required. The antigens most commonly employed are: Nuclear Antigen detected by Ki67 antibody [13], DNA polymerase alpha [14], Proliferating Cell Nuclear Antigen (PCNA/Cyclin) [15], p105 Nuclear Antigen [16], Nucleolar Antigens [17], Mitosing Cell Antigen detected by C5F10 antibody [18], and Transferrin Receptor (CD 71) [13]. They are used in accordance with different methods and differ in their specificity with regard to one or more phases of the cell cycle.

Ki67 is a monoclonal antibody that identifies a nuclear antigen expressed in late G1-, S-, G2- and M-phase cells, but not in early G1 and resting cells. Its main drawback is that it can only be used on frozen sections.

DNA polymerase alpha is the main enzyme associated with DNA replication. Intense nuclear immunoreactivity is observed in G1-, G2- and S-phase cells, but none in G0; cytoplasmic reactivity is observed during the M-phase. Immunohistochemical detection is confined to frozen sections postfixed with 3% paraformaldehyde.

PCNA is the auxiliary protein of DNA polymerase delta and plays a critical role in the initiation of proliferation. Cells in G0 and G1 do not express significant amounts of PCNA, whereas a progressive increase is displayed by those in advanced G1-phase and even more so in the S-phase. In G2 and M, nuclear levels settle at values between those in G1 and in S. PCNA is a stable protein and is not degraded during the passage to the non-cycling compartment; 40% is still present at least 48 hours after entry into G0. There are two PCNA subpopulations in the nucleus: 30% of nuclear PCNA is tightly associated with DNA replication sites. Its antigen determinants are preserved by fixation either in formalin or methanol, and it is specifically correlated with the S-phase. A second PCNA pool diffuses into the nucleoplasm. It is also present in cells that have been in G0 for a short time and can only be preserved by fixation in formalin. In the normal intestinal mucosa, therefore, BrdU-index coincides with the PCNA-index in methanol-fixed biopsies, whilst it is inferior in formalin-fixed biopsies. These results refer to detection of PCNA with 19A2 MAb. A more recent monoclonal antibody (PC10) [19] offers no more than partial reduction of the effect of fixation on PCNA immunoreactivity.

The specific efficacy of these new markers in the mapping of human colon mucosa proliferation awaits final determination. Our unpublished findings indicate that the distribution of PCNA+ and BrdU+ cells is virtually identical in the crypts of normal control subjects. Both Ki67+ cells and DNA polymerase alpha+ cells occupy higher levels than BrdU+ cells. The BrdU-LI and the PCNA index are very close, whereas the Ki67 and DNA polymerase alpha indices are much higher. These data are consistent with the differences in the size of the populations labeled by each antibody: S-phase cells by BrdU and PCNA; G1+S+G2 by Ki67 and DNA polymerase alpha. In the mucosa of subjects at high risk for colorectal carcinoma, marked BrdU+ cell distribution abnormalities (especially the shift of the proliferative zone to the crypt surface) are much less evident with the other markers, including PCNA. Proliferation profiles for groups at risk which are readily distinguishable from the normal in statistical terms when determined by BrdU uptake lose their significance when determined on the strength of Ki67, PCNA or DNA polymerase alpha positivity. Immunohistochemical detection

of these proliferation-associated antigens, therefore, is not sensitive enough to identify at least some of the crypt proliferation distribution abnormalities. It does, however, provide essential information in more advanced stages of intestinal carcinogenesis, as will be shown later.

Immunohistological Analysis

Quantification of proliferation levels and distribution requires the establishment of indices describing proliferation in each crypt sector as a supplement to the total labeling index (TLI), which expresses the global proliferative activity. For this purpose, each hemicrypt is divided into three [20] or ten [21] longitudinal compartments. Our preference is for the recent tendency to distinguish five compartments, ranging from No. 1 at the base to No. 5 at the mouth of the crypt [22,23]. It has been shown that the main events in cytokinetic derangement occur in the top 40% of the crypt. A division that specifically takes this segment (compartments 4 and 5) into account is thus acceptable, whereas some minimal alterations may be overlooked in the three-compartment model. In addition to the TLI, the LI of each compartment (LI 1–5), the percentage of compartments containing at least one labeled cell (P 1–5), and the percentage distribution of labeled cells in the five compartments (C 1–5) are calculated. This system is certainly more laborious than Lipkin's determination of the Φ_h value [21]. In our view, however, it provides a more detailed description of the state of proliferation in the base of the crypt (compartments 1 and 2).

CYTOPROLIFERATION IN NORMAL MUCOSA

The cytokinetic profile of a control population of about 600 subjects was determined from the mean values of the 16 parameters mentioned earlier. Reference was made to the rectal mucosa following the observation that only slight and nonsignificant differences exist for the TLI solely in the more proximal segments of the colon. The influence of demographic factors on rectal mucosal proliferation is known [24]. In our series, slight hyperproliferation with expansion of the proliferative zone in subjects over 60 was noted solely in subgroups originating from and/or resident in specific geographical regions. It may be the expression of nutritional differences and it may also be linked to higher incidence of colorectal carcinoma in the elderly. Since the more complex relations between demographic factors and proliferation have not yet been unravelled, careful matching of controls is advisable to enable even minimal proliferation deviations in groups at risk to be detected.

The influence of the tissue microenvironment on epithelial renewal is revealed through proliferation alterations in crypts beside follicular structures of gut-associated lymphoid tissue (the so-called "lymphoepithelial complexes"). These alterations substantially take the form of slight expansions of the proliferative compartment with a lifting of the cutoff zone involving the 3–5 crypts close to the follicle. They occur infrequently (1/20 complexes) in subjects not older than 40 and probably reflect physiological modulation of epithelial proliferation on the part of the lymphoreticular tissue.

EPITHELIAL CELL PROLIFERATION IN INFLAMMATORY DISORDERS

Growth Fraction and Cell Cycle Time modifications are the main compensation mechanisms ensuring cell renewal in response to both physiological and pathological stimuli, such as meals and increased cell loss [1]. The human intestinal mucosa is extremely sensitive to inflammation-induced changes in its microenvironment, as we found in 180 cases of colorectal inflammatory disorders of varying etiology. Paradigmatic epithelial proliferation changes occur in self-limited colitis, a clinically and pathologically distinct condition marked by segmentary inflammation of the colon that is self-resolving within 3–5 weeks. In the segment concerned, the proliferative compartment expands by upward movement of the cutoff position, even as far as the surface of the mucosa. The labeled cells are either uniformly distributed along the crypt or more densely concentrated in its deep third. This state of hyperproliferation is reflected in increases in the TLI and all compartment LIs. P 4–5 are also increased, whereas these parameters are unchanged or reduced in deep compartments.

Sequential biopsies show that the proliferation profile returns to normal some weeks after clinical and endoscopic healing. Similar, transient changes are observed in a small percentage of cases of Crohn's disease, acute radiation injuries, and colitis with a specific etiology (Tuberculous, Yersinia, Schistosoma), and occasionally in collagenous colitis. In microscopic colitis, cell renewal is unchanged, the inflammatory infiltrate is somewhat bland and superficial, and does not involve the microenvironment immediately surrounding the stem cells and the proliferative compartment at the base of the crypt.

Ulcerative colitis is also accompanied by hyperproliferation. Here the kinetic alterations are confined to the mucosa of the diseased tracts; this fact invalidates the hypothesis of a proliferative abnormality of the entire colorectal mucosa prior and predisposing to the disease itself [25]. Hyperproliferation, albeit reduced, persists during remission and continuing subclinical, subhistological mucosal damage may thus be postulated [25]. A totally different proliferation alteration has been described in some ulcerative colitis subgroups [26] and as a late feature of irradiated mucosa [27] which takes the form of gradual displacement of the major DNA synthesis zone to the intermediate and surface portions of the crypt. This upward shift (Deschner and Masken's "Stage II abnormality" [28]) is recognized as S-phase cells clumping in compartments 3, 4, 5, coupled with partial or total depletion of proliferation in compartments 1 and 2. The TLI is unchanged, whereas LI 3, 4, 5 and P 3, 4, 5 are increased. There is also a peculiar inversion of the ratio between C 1, 2 and C 4, 5. This abnormality is observed in some groups of ulcerative colitis patients with a high risk of cancer and after radiotherapy of the pelvis, itself accompanied by a higher risk of subsequent rectal carcinoma. There is also evidence that an upward shift is an early step in large bowel carcinogenesis [29] and can thus be used as a reliable biomarker.

MUCOSA CELL PROLIFERATION IN COLORECTAL NEOPLASIA

Despite the focal nature of dysplastic or carcinomatous lesions, substantial cell proliferation changes are observed in the entire, albeit histologically and endoscopically normal, bowel mucosa in colorectal neoplasia. The TLI and compartment LIs are slightly higher in the mucosa near the tumor site than in more distant sectors, though even here the proliferation profile is not normal [30]. The most frequent kinetic alteration in the mucosa of patients with non-familial neoplasia is hyperproliferation progressively increasing in severity from adenoma to adenocarcinoma alone and with adenoma. Furthermore, proliferative activity is correlated with adenoma size: if it is less than 1 cm, TLI and LI 1–5 are similar to the controls and significantly lower than in subjects with larger tumors, though the distribution of proliferating cells along the crypt axis is the same in both groups [23]. From the kinetic standpoint, these results indicate a different biology of small as opposed to large adenomas with respect to the risk of metachronous lesions. Proliferation decreases as a function of the length of the polyp-free colon state (significantly after 2 years). The chronology of the neoplastic lesions, too, has no influence on the distribution of proliferating cells [23].

Normalization of proliferation after endoscopic and/or surgical establishment of a negative colon is an important phenomenon to be borne in mind when assessing the soundness of hyperproliferation as an intermediate biomarker in chemoprevention studies. Radical changes in eating habits or substantial chemistry modifications in the lumen of a polyp-free colon could be responsible for this phenomenon. Direct tumor involvement in the maintenance of hyperproliferation via an autocrine mechanism is an equally legitimate hypothesis. Our preliminary data, in effect, show an appreciable correlation between the mucosal TLI and the percentage of cells immunoreacting to alpha-type Transforming Growth Factor in the corresponding adenomas, as well as abnormal expression of Epidermal Growth Factor receptors in the mucosal S-phase cells of patients with large bowel neoplasia [30].

The upward shift of the major zone of DNA synthesis accompanies hyperproliferation in the mucosa of subjects with colorectal adenomas or adenocarcinomas. It is more frequent in adenomas with a high rather than a low degree of dysplasia, and more pronounced in adenomas plus adenocarcinoma than in either form alone

[23]. A comparison has recently been made of the mucosal proliferation profiles of subjects with non-familial colorectal neoplasia and subjects with familial aggregation of colon adenomas and cancer, previously matched for clinical and pathological features of neoplasia. Hyperproliferation was highest in the group with non-familial colorectal neoplasia, and the stage II abnormality was most marked in the group with familial aggregation of colorectal neoplasia [30]. In short, colorectal neoplasia is associated with complex changes in cell proliferation throughout the mucosa of the large intestine that can be broken down into elementary alterations, i.e., hyperproliferation and the stage II abnormality, open to use as separate biomarkers of different levels of the risk of colorectal carcinoma, since they are independent and differently correlated with a variety of clinical and pathological features. Moreover, hyperproliferation is associated with ambient and microenvironmental factors and is particularly evident in non-familial neoplasia, whereas the stage II abnormality is probably the expression of endogenous and genetic risk factors.

COLORECTAL CARCINOMA: MORPHOGENETIC PATHWAYS AND CYTOKINETIC ABNORMALITIES

One of the most interesting carcinogenesis models is the so-called adenoma-carcinoma sequence of the large bowel, i.e., development of malignancy in a dysplastic focal precursor [31]. As Lightdale and Lipkin have demonstrated in vivo [29] the earliest stage in the genesis of an adenoma is an upward shift of the proliferative zone, followed by retrograde migration of S-phase cells to the base of the crypt and hence the genesis of a single-crypt adenoma. Repeated budding and ramification then lead to a microscopic and eventually macroscopic neoplasm. The cell proliferation profiles of the adenomatous crypt recapitulate the proliferative abnormalities of the morphogenesis. Irrespective of the degree of dysplasia, in fact, there is expansion throughout the crypt and/or concentration of proliferative activity in the surface segments [32]. Little is known about cell proliferation changes in two other less frequent morphogenetic sequences of colon carcinoma, namely "de novo" adenocarcinoma [33] and adenocarcinoma arising from flat adenoma [34]. As its name implies, "de novo" adenocarcinoma arises as such in the absence of dysplastic precursors. This morphogenetic pathway is rare in man, though frequently observed in the experimental animal. Colorectal carcinoma induced by 1,2-dimethylhydrazine in CF1 mice is a model in which the development of multiple neoplastic lesions is preceded by adenomas, itself preceded by an upward shift of the proliferation zone. BD IX rats treated in the same way develop microinvasive "de novo" carcinomas, with a downward shift from the normal intermediate location of the proliferation zone. The shift direction, therefore, may be supposed to determine the features of a morphogenetic sequence in accordance with the hypothesis of Deschner and Maskens [28]. In our experience, "de novo" adenocarcinoma was not associated with significant changes in mucosal cell proliferation, and a downward shift can perhaps be postulated in man. This, of course, would not be evident as such, since the proliferative compartment is usually located at the base of the human crypt. Adenocarcinoma arising from flat adenoma is another possible sequence. The endoscopic and histological features of its precursor are clear. In our series, this form was marked by a very distinct stage II abnormality, whereas the TLI increase was small. This underscores the importance of hereditary factors in the genesis of this type of adenocarcinoma.

CELL PROLIFERATION IN THE ADENOMA-CARCINOMA SEQUENCE

Proliferative activity increases progressively from the normal mucosa to adenomas with low- and then high-grade dysplasia, where the mean BrdU LI is comparable to that observed in advanced cancer [32]. From the kinetic standpoint these results substantiate the histogenetic theory that colorectal carcinoma arises from adenomas through consecutive steps of increasing severity of dysplastic alterations. (In other types of polyps, such as hyperplastic polyps, the LI is similar to that of normal mucosa and the proliferation zone is strictly confined to the base

of the crypt. There is an alteration in cell differentiation and migration along the crypt, but proliferation itself is unchanged [32]). The Ki67 and DNA polymerase alpha indices run parallel to the BrdU LI. Both the PCNA index and the BrdU LI gradually increase as a tumor progresses, but the differences between the means of these two indices become more pronounced from adenomas with low-grade dysplasia to advanced cancer. There is a close correlation between these indices in normal mucosa and low-grade dysplasia adenomas, but not in high-grade dysplastic adenoma and advanced cancer [35]. PCNA expression is thus on a par with specific S-phase markers in normal tissue, whereas during tumor progression it is correlated with both kinetic activity and neoplastic deregulation in the synthesis of proliferation-associated antigens. PCNA, indeed, may play a non-replicative role in the nucleus [36,37], since its concentration exceeds that needed for replication, while recent work has shown that it may also be involved in the repair of DNA.

Early cancer is a key point in the progression of large bowel tumors. "Adenoma containing invasive carcinoma" (ACIC) is the earliest form of clinically relevant colorectal cancer in most patients. As generally defined, ACIC comprises a carcinoma that invades the submucosa, but not the muscularis [38]. The PCNA, Ki67 and DNA polymerase alpha indices separately evaluated in selected fields of histological ACIC sections corresponding to low-grade and high-grade dysplasia and early cancer agree in indicating a decrease in proliferative activity in early cancer compared with high-grade dysplasia [39]. This phenomenon may perhaps be ascribable to serious alterations in the submucosal microenvironment brought about by carcinomatous invasion, particularly stromal desmoplasia resulting in disconnection of the vascular network. The influence of vascular support on proliferation [40], in fact, has been demonstrated in both human disorders and animal models.

FUTURE PROSPECTS

An indispensable requirement is the acquisition of data and a detailed knowledge of the correlations between elementary changes in mucosal cell proliferation and the demographic, environmental, endogenous and genetic factors concurrently involved in the genesis of large bowel carcinoma. The aim is to identify a panel of biomarkers specifically directed to the detection of individual risk factors.

Another fundamental need is characterization of the differentiative and phenotypical aspects (oncoproteins, growth factors and homologous receptors, etc.) of cell populations with proliferation abnormalities, both in the mucosa of groups at risk and in the more advanced stages of carcinogenesis (early cancer). Here the objective is to define the chronological and biological sequences of cytokinetic derangement in tumor progression, and identify targets within such sequences against which chemoprevention could be usefully directed.

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