Journal of Cellular Biochemistry, Supplement 16G:73-78 (1992)

Cell Proliferation Biomarkers in the Gastrointestinal Tract

Guido Biasco, Gian Maria Paganelli, Mario Miglioli, and Luigi Barbara

Department of Clinical Medicine and the "Giorgio Prodi" Center for Cancer Research, University of Bologna, 40138 Bologna, Italy

Abstract Measurements of epithelial cell proliferation in the mucosa of the gastrointestinal tract pointed out the existence of cell kinetic abnormalities which can be involved in the first steps of carcinogenesis. In particular, an increase in the cell proliferation rate and an abnormal distribution of proliferating cells were found both in animals exposed to carcinogens and in human subjects at high risk of gastrointestinal cancer. In some diseases which predispose to cancer (*i.e.*, chronic atrophic gastritis, hereditary gastrointestinal cancer, sporadic colorectal neoplasia, chronic ulcerative colitis) we observed an expansion of the proliferative compartment even when the mucosa was not affected by morphological abnormalities. This proliferative feature seems to be associated with the presence of defects in cell differentiation. The abnormality is well detected by the histological examination of the proliferative pattern using microautoradiography after incorporation of tritiated thymidine, or using immunohistochemistry after bromodeoxyuridine uptake. The literature, and our own results, indicate that the search for abnormalities of epithelial cell proliferation can be useful in studying the earliest mechanisms leading to gastrointestinal cancer, in detecting subjects at high cancer risk, and for pilot chemoprevention studies using these abnormalities as intermediate biomarkers of gastrointestinal cancer risk. © 1992 Wiley-Liss, Inc.

Key words: bromodeoxyuridine, cell proliferation, chemoprevention, chronic atrophic gastritis, colorectal adenomas, experimental carcinogenesis, gastrointestinal cancer, intermediate biomarker, proliferative compartment, tritiated thymidine, ulcerative colitis

According to the current hypothesis, the progression from normal gastrointestinal mucosa to cancer is a stepwise process. The occurrence of abnormalities and the rate of progression from one step to another are under the influence of genetic and environmental factors. On the other hand, the beginning of the process leading to neoplasia does not necessarily mean the development of cancer. We can say that the probability of developing cancer progressively decreases as neoplastic transformation goes on. An example is colorectal adenomas, which are the main preneoplastic lesions of the large bowel but become malignant to a small extent (1).

EARLY PRENEOPLASTIC CHANGES

Biological errors can represent the earliest abnormalities in this process. These errors can be present even in the absence of morphological alteration of the gastrointestinal mucosa, but are maintained in the following steps. For example, an error of the control of cell growth occurs very early in apparently normal mucosa and generally persists up to the development of neoplasia (2).

The distribution of early mucosal changes is probably more widespread than advanced lesions (3). Moreover, some evidence indicates that early lesions could revert to normal (4). Above all, biological changes of the mucosa of the gastrointestinal tract can be of interest as intermediate biomarkers of cancer risk in studies aimed to test the effect of xenobiotics with possible oncogenic or anti-oncogenic properties (5).

An abnormal cell proliferation is an early biological alteration within the carcinogenesis process. This hypothesis is supported by several studies (6-10). However, some points must be expanded and clarified: 1) which cell kinetics alterations are related to gastrointestinal cancer; 2) how can we study these alterations; 3) what are the clinical implications.

Address reprint requests to Guido Biasco, M.D., Istituto di Clinica Medica e Gastroenterologia, Policlinico S. Orsola, Via Massarenti 9, 40138 Bologna, Italy

© 1992 Wiley-Liss, Inc.

CELL KINETICS ALTERATIONS RELATED TO GASTROINTESTINAL CANCER

The normal cell proliferation pattern of the gastrointestinal tract shows particular features.

In the gastric mucosa, proliferating cells are located mainly in the neck of the glands. From this stem cell area, cells migrate towards the surface. During their trip they progressively loose their proliferative capacity and at the same time they undergo differentiation. In the gastric body and fundus, some cells migrate downwards and become parietal cells. Peptic cells and endocrine cells seem to have an autonomous replicative cycle (11).

In the colon, the cell proliferative area is located in the lower two-thirds of the glands. Newborn cells migrate upwards and become mature elements with absorption or mucus secretion capacity (12).

In animal models of gastric carcinogenesis, during treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), the number of proliferating cells per pyloric crypt column and the width of the proliferative zone greatly increase. Therefore, the number of epithelial cells in each column and the thickness of the pyloric mucosa increase significantly (13).

In ACI rats, which are susceptible to gastric chemical carcinogenesis, the administration of MNNG induces an increase of cell proliferation rate and an upward shift of the proliferative compartment before development of cancer (14). In resistant Buffalo rats, pyloric cells do not increase after treatment with MNNG. This experiment suggests not only the existence of genetic susceptibility to cancer development, but also that an increase of proliferating cells pool is one of the earliest changes.

Similar considerations can be drawn from studies on colon carcinogenesis. In an early study by Thurnherr and Lipkin (15), CF1 mice were given weekly injections of dimethylhydrazine (DMH). This treatment induced colonic carcinomas in 90% of the animals after 86 days. The autoradiographic analysis of normal-appearing crypt after injection with tritiated thymidine showed that after 45 days of treatment the proliferating cell compartment expanded toward the superficial mucosal layers as compared to control animals. However, the overall number of proliferating cells was not changed. After 87 days of treatment, the total number of proliferating cells was also increased.

On the basis of these findings, experimental studies suggest that the action of carcinogens can lead to three different proliferative features: at first, an expansion or an upward displacement of the proliferative compartment; then an increase of the proliferating cell pool; and finally an upward shift of the proliferative compartment together with an increased number of proliferating cells. These proliferative abnormalities could be considered as precancerous changes. They can appear simultaneously or separately in the mucosa exposed to carcinogens.

Nevertheless, some problems arise if we want to use measurements of cell proliferation as a marker of cancer risk in humans. As a matter of fact, the biological significance of these abnormalities may be different in different circumstances. For example, studies on the stomach demonstrated that physiological or pathological non-carcinogenic factors can induce changes in the proliferative pattern of the mucosa (16). Physiological stimuli such as food ingestion lead to the same effect (17). In this circumstance, this is due to a loss of epithelial cells from the mucosal surface.

Similar observations are derived from studies on the colonic mucosa. Lehy and coworkers (18) showed that colon cleansing preparations, such as laxatives or enemas, induce an increase of the proliferating cell pool and an increase of the proliferative compartment size in the colorectal mucosa. The effect is probably due to an exfoliation of cells from the mucosal surface.

The same mechanism is involved during acute inflammation of the mucosa. For example, in active ulcerative colitis we observe an increase of the proliferating cell pool (19). Expanded studies on this disease show that two main kinetic patterns are related to active inflammation. The first one is represented by an increased number of proliferating cells without any change of the proliferative compartment. In autoradiographical or immunohistochemical studies after incubation in vitro with tritiated thymidine or bromodeoxyuridine (BrdU), this change is expressed by an increased labeling index. An increase of the labeling index can be considered as a compensatory mechanism to an enhanced cell exfoliation from the mucosal surface, which occurs in active phases of the disease. When the colitis returns to a quiescent stage, the labeling index reaches values similar to those observed in the normal mucosa of healthy controls (19).

The biological mechanisms which underlie an increase of proliferating cells could be a recruitment of cells from G_0 phase and a decrease of the duration of the G_1 phase (20).

Studies carried out with the tritiated thimidine-double labeling technique demonstrated that the duration of S phase is not influenced by the cell turnover rate (19). Therefore, the increased number of proliferating cells is an expression of an increased production of new elements.

However, in active ulcerative colitis the increase of replicating cells is often associated with an expansion of the proliferative compartment (21). This pattern could be also the consequence of an increased cell loss and a very fast migration of cells toward the surface.

Another explanation is that under replicative stimuli, a single stem unit undergoes a larger number of proliferative cycles (20). This leads to an increase of the size of the proliferative compartment. Also the expansion of the proliferative compartment can revert to normal in quiescent stages of the disease (19).

Therefore, inflammatory exfoliating agents can lead to at least two proliferative changes similar to those induced by carcinogens: an increase of proliferating cell pool with or without an increase of the size of the proliferative compartment. In this case, these cell kinetics changes must be considered physiological responses to a damaging agent.

We cannot exclude that these physiological mechanisms are cancer risk factors themselves. As mentioned above, the increase of proliferating cells could lead to an increased susceptibility to carcinogens and to precancerous cell kinetics defects.

For example, In chronic gastritis we can frequently observe an expansion of the proliferative compartment (22). In chronic ulcerative colitis, an expansion of the proliferative compartment could persist even in absence of active inflammation and increased cell turnover (8). This abnormal location of proliferating cells can be related to a deregulation of cell proliferation and differentiation control and not to a compensatory mechanism to cell loss.

Therefore, chronic stimulation of cell proliferation could induce stable changes with precancerous significance. This effect could be due to an increase of proliferating cells susceptible to the action of carcinogens.

We must take into account these mechanisms in the biological history of the neoplastic development. However, if we wish to use cell kinetics alterations as biomarkers of risk, we have to exclude, whenever is possible, those changes which seem not to be necessarily due to the action of carcinogens, even if they could be a risk factor.

METHODS TO STUDY CELL KINETICS

The choice of the method to study cell kinetics is a very important issue. Several techniques are available to study cell kinetics in the gastrointestinal mucosa, and each one of them explores different aspects of cell proliferation.

We attempted to evaluate the correlation between various techniques in normal rectal mucosa of patients at high risk of colon cancer. For example, we have found a good correlation between BrdU-labeling and the mucosal activity of the enzyme ornithine decarboxylase (23), and between tritiated thymidine labeling and the percentage of cells in "S" phase determined by flow cytometry in frozen and in formalin-fixed specimens (24).

However, the correlation concerns only BrdU and thymidine labeling indices. As mentioned above, this parameter can be influenced by physiological events, such as active inflammation or increased cell loss, and perhaps it is not the most reliable proliferative marker of cancer risk. Moreover, methods utilizing isolated cells or homogenized tissue do not allow us to evaluate the pool of cells which are examined nor the distribution of proliferating cells within the tissue. As a matter of fact, an inflamed mucosal specimen is highly infiltrated with inflammatory cells which can interfere with the measurement of epithelial cell proliferation. For these reasons, the most reliable methods are probably those utilizing tissue culture, such as tritiated thymidine or BrdU uptake.

Using these methods, it is possible to study the earliest proliferative patterns which can be related to cancer: 1) an expansion of the proliferative compartment without increase of the labeling index; 2) an expansion of the proliferative compartment with increase of the labeling index but without acute inflammation of the tissue; 3) an upward shift of the area of maximal proliferation.

We compared the frequency of distribution of proliferating cells in apparently normal rectal mucosa of hospital controls and patients with neoplastic or preneoplastic colorectal diseases (10). The values of maximal differences between patients and controls are located in the upper two-fifths of the crypts. In this area, patients show an excess of proliferating cells. These results are similar to those observed in patients with hereditary predisposition to colorectal cancer. The frequency of occurrence of labeled cells in the upper two-fifths of the crypts has been defined as the ϕh value by Lipkin et al. (25).

These observations sustain the hypothesis of a precancerous significance of this cell proliferation pattern. The ϕh value seems to be a good biomarker of cancer risk in population groups. However, its reliability as a marker of the individual cancer risk level has to be discussed. For example, in patients with adenomas or cancer of the large bowel, there is a large overlap of ϕh values as compared to the controls (9, 10). Since this kind of study has been carried out on randomized biopsies of apparently normal mucosa, the results can be the consequence of a patchy distribution of the proliferative abnormalities within the colorectal mucosa. Therefore, biopsy sampling could have failed to collect areas with this abnormality. On the other hand, we could speculate that subjects without any lesion of the large bowel but showing high ϕh values could develop colon cancer in the future. Unfortunately, the lack of prospective studies does not allow us to verify this hypothesis. However, despite these limitations we have some data sustaining the hypothesis that high ϕh values could identify patients at ultra-high risk within a given population at intermediate risk.

For example, among patients affected by chronic atrophic gastritis, we found subjects with a high frequency of labeled cells in the upper portions of gastric glands (22). Similar results were obtained by Lipkin et al. (26). In particular, subjects with an abnormal expansion of the proliferative compartment also showed the presence in their mucosa of fetal antigens. These antigenic abnormalities were absent in patients affected by chronic atrophic gastritis without any abnormal expansion of the proliferative compartment, such as in normal subjects (26).

We obtained similar results on rectal biopsies of patients with ulcerative colitis (27). Patients with high ϕh values showed antigenic abnormalities of their mucosa, suggesting a loss of cell differentiation. In a more recent study, some patients with quiescent ulcerative colitis showed particularly high ϕh values in their rectal mucosa (8). The distribution of patients according to their ϕh suggested the existence of two groups of patients, both with ϕh higher than controls. This abnormality does not seem to be related to the duration of the disease. Patients with higher ϕh values showed also an abnormal antigenic expression of cytoskeletal-associated proteins (28). This result is an additional support to the hypothesis that high ϕh values could express a particularly high derangement of cell proliferation and differentiation perhaps with precancerous significance.

Finally, a recent work on patients operated on for colon cancer shows that patients with high ϕh values in the rectal mucosa are prone to develop new adenomas (29). Patients with values similar to the controls do not seem to have this propensity.

CLINICAL IMPLICATIONS

In summary, studies in man suggest that despite some limitations, cell proliferation abnormalities could be proposed as intermediate biomarkers of gastrointestinal cancer risk. These biomarkers could be useful to evaluate both the individual level of cancer risk and, perhaps more important, the effect of exogenous or endogenous factors in human carcinogenesis.

As far as colon is concerned, cell proliferation was used to assess the action of bile acids on colorectal mucosa. Studies in the literature suggest that fecal bile acids are able to stimulate cell turnover rate. The pathogenetic mechanism leading to an increased cell proliferation is not yet established (30, 31). We must consider that the increased cell turnover rate could either lead to an increased number of cells susceptible to the action of carcinogens or to amplify cell defects induced by carcinogens. The latter action must be regarded as a promotion effect.

From a preliminary analysis of part of the data of a case-control study sponsored by the European Cancer Prevention Organization (ECP) on patients with colon adenoma or cancer, we did not find any correlation between the concentration of fecal bile acids and cell kinetics parameters. On the contrary, we found a significant correlation, even if not very strong, between ϕh value and the ratio of lithocolic/deoxycholic acid (32). It is interesting to point out that this ratio is considered a marker of colon cancer risk by other authors (33).

Finally, we found an inverse correlation between both the labeling index and ϕh and the percentage of neutral sterols of plant origin in the feces (32). Some of these, especially beta-sitosterol, are known to be inhibitors of cholesterol absorption. This could influence the synthesis of chenodeoxycholic acid, because lithocholic acid production is under the influence of dietary cholesterol input.

Starting with the first preliminary experience with calcium by Lipkin and Newmark (4), several studies were carried out on the effect of several possible chemopreventive compounds on cell

proliferation. We recently evaluated the effect of oral supplementation with vitamins A, C and E on rectal cell proliferation of 20 patients affected by adenomas of the large bowel, after complete endoscopic removal of polyps (34). These vitamins have several effects in models in vivo and in vitro, such as an inhibition of the formation of nitroso compounds in the GI lumen, a free radical scavenger effect, and modulation of cell proliferation and differentiation. We observed that the frequency of occurrence of labeled cells in the upper layers of the mucosa was significantly decreased after three and six months of treatment with vitamins. This suggests that these compounds could have a chemopreventive action on colorectal cancer.

Obviously, these results are still preliminary. Further work is needed to clarify the mechanisms regulating cell proliferation and differentiation, as well as the substances able to influence it. At present, we can conclude that despite some limitations and the lack of definitive validation (35), the use of biomarkers of cell proliferation as intermediate endpoints in chemoprevention trials of gastrointestinal cancer is worthwhile and promising.

BIBLIOGRAPHY

1. Muto T, Bussey HJR, Morson BC: The evolution of cancer of the colon and rectum. Cancer 1975; 36: 2251-2270
2. Lipkin M: Phase 1 and phase 2 proliferative lesions in diseases leading to colon cancer. Cancer 1974; 34: 878-888
3. Terpstra OT, van Blankenstein M, Dees J, Eilers GAM: Abnormal pattern of cell proliferation in the entire colonic mucosa of patients with colon adenoma or cancer. Gastroenterology 1987; 92: 704-708
4. Lipkin M, Newmark H: Effect of added dietary calcium on colonic epithelial cell proliferation in subjects at high risk for familial colon cancer. N Engl J Med 1985; 313: 1381-1384
5. Lipkin M: Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. Cancer Res 1988; 48: 235-245
6. Ponz de Leon M, Roncucci L, Di Donato P, Tassi L, Smerieri O, Amorico MG, Malagoli G, De Maria D, Antonioli A, Chahin NJ, Perini M, Rigo G, Barberini G, Manenti A, Biasco G, Barbara L: Pattern of epithelial cell proliferation in colorectal mucosa of normal subjects and of patients with adenomatous polyps or cancer of the large bowel. Cancer Res. 1988; 48: 4121-4126
7. Risio M, Coverlizza S, Ferrari A, Candelaresi GL, Rossini FP: Immunohistochemical study of epithelial cell proliferation in hyperplastic polyps, adenomas, and adenocarcinomas of the large bowel. Gastroenterology 1988; 94: 899-904
8. Biasco G, Paganelli GM, Miglioli M, Brillanti S, Di Febo G, Gizzi G, Ponz de Leon M, Campieri M, Barbara L: Rectal cell proliferation and colon cancer risk in ulcerative colitis. Cancer Res 1990; 50: 1156-1159
9. Risio M, Lipkin M, Candelaresi GL, Bertone A, Coverlizza S, Rossini FP: Correlations between rectal mucosa cell proliferation and the clinical and pathological features of nonfamilial neoplasia of the large intestine. Cancer Res 1991; 51: 1917-1921
10. Paganelli GM, Biasco G, Santucci R, Brandi G, Lalli AA, Miglioli M, Barbara L: Rectal cell proliferation and colorectal cancer risk level in patients with non-familial adenomatous polyps of the large bowel. Cancer 1991; 68: 2451-2454
11. Willems G, Lehy T: Radioautographic and quantitative studies on parietal and peptic cell kinetics in the mouse. Gastroenterology 1975; 69: 416-426
12. Lipkin M, Sherlock P, Bell B: Cell proliferation kinetics in the gastrointestinal tract of man. II. Cell renewal in stomach, ileum, colon, and rectum. Gastroenterology 1963; 45: 721-729
13. Deschner EE, Tamura K, Bralow SP: Early proliferative changes in rat pyloric mucosa induced by N-methyl-N'-nitro-N-nitrosoguanidine. Front Gastrointest Res 1984; 4: 25-31
14. Ohgaki H, Tomihari M, Sato S, Kleihues P, Sugimura T: Differential proliferative response of gastric mucosa during carcinogenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine in susceptible ACI rats, resistant Buffalo rats, and their hybrid F_1 cross. Cancer Res 1988; 48; 5275-5279
15. Thurnherr N, Deschner EE, Stonehill EH, Lipkin M: Induction of adenocarcinomas of the colon in mice by weekly injections of 1,2-dimethylhydrazine. Cancer Res 1973; 33: 940-945
16. Miller LR, Jacobson ED, Johnson LR: Effect of pentagastrin on gastric mucosal mucosal cells grown in tissue culture. Gastroenterology 1973; 64: 254-267
17. Willems G, Vansteenkiste Y, Smets P: Effect of food ingestion on the cell proliferation kinetics in the canine fundic mucosa. Gastroenterology 1971; 61: 323-327
18. Lehy T, Abitbol JL, Mignon M: Influence de la préparation rectale par lavement sur la prolifération cellulaire dans la muqueuse rectale normale de l'homme. Gastroenterol Clin Biol 1984; 8: 216-221
19. Biasco G, Santini D, Marchesini F, Di Febo G, Baldi F, Miglioli M, Barbara L: Kinetics of the mucous cells of the rectum in patients with chronic ulcerative colitis. Front Gastrointest Res 1979; 4: 65-72
20. Wright NA, Alison M: The biology of epithelial cell populations. Vol.2. Oxford: Clarendon 1984; 743-778
21. Biasco G, Azzaroni D, Miglioli M, Minarini A, Gizzi G, Di Febo G, Rusticali AG, Barbara L: Analysis of rectal cell kinetics in patients with active ulcerative

colitis. Ital J Gastroenterol 1985; 17: 94-95
22. Biasco G, Paganelli GM, Brillanti S, Lalli AA, Brandi G, Terranova A, Miglioli M, Barbara L: Cell renewal and cancer risk of the stomach: analysis of cell proliferation kinetics in atrophic gastritis. Acta Gastroenterol Belg 1989; 52: 361-366
23. Paganelli GM, Saccoccio G, Brandi G, Bellentani S, Santucci R, Armocida C, Ponz de Leon M, Biasco G: Correlation between bromodeoxyuridine labelling and ornithine decarboxylase levels in normal rectal mucosa of patients with colorectal adenoma. Cancer Lett 1991; 59: 221-224
24. Paganelli GM, Lalli E, Facchini A, Biasco G, Santucci R, Brandi G, Barbara L: Flow cytometry and thymidine labelling in normal rectal mucosa of patients at high risk of colorectal cancer. Eur J Basic Appl Histochem 1991; 35: 266
25. Lipkin M, Blattner WE, Fraumeni JF Jr, Lynch HT, Deschner EE, Winawer S: Tritiated thymidine (ϕp, ϕh) labeling distribution as a marker for hereditary predisposition to colon cancer. Cancer Res 1983; 43: 1899-1904
26. Lipkin M, Correa P, Mikol YB, Higgins PJ, Cuello C, Zarama G, Fontham E, Zavala D: Proliferative and antigenic modifications in epithelial cells in chronic atrophic gastritis. J Natl Cancer Inst 1985; 75: 613-619
27. Biasco G, Lipkin M, Minarini A, Higgins P, Miglioli M, Barbara L: Proliferative and antigenic properties of rectal cells in patients with chronic ulcerative colitis. Cancer Res 1984; 44: 5450-5454
28. Paganelli GM, Lipkin M, Higgins PJ, Biasco G, Brandi G, Miglioli M, Barbara L: Cell proliferation and differentiation abnormalities in ulcerative colitis. Gastroenterology 1990; 98: A302
29. Scalmati A, Roncucci L, Ghidini G, Biasco G, Ponz de Leon M: Epithelial cell kinetics in the remaining colorectal mucosa after surgery for cancer of the large bowel. Cancer Res 1990; 50: 7937-7941
30. Craven PA, Pfanstiel J, Saito R, DeRubertis FR: Relationship between loss of rat colonic surface epithelium induced by deoxycholate and initiation of the subsequent proliferative response. Cancer Res 1986; 46: 5754-5759
31. DeRubertis FR, Craven PA, Saito R: Bile salt stimulation of colonic epithelial proliferation. Evidence for involvement of lipoxygenase products. J Clin Invest 1984; 74: 1614-1624
32. Biasco G, Paganelli GM, Owen RW, Hill MJ, and the ECP Colon Cancer Working Group: Faecal bile acids and colorectal cell proliferation. Eur J Cancer Prevent 1991; 1 (suppl. 2): 63-68
33. Owen RW, Thompson MH, Hill MJ, Wilpart M, Mainguet P, Roberfroid M: The importance of the ratio of lithocholic to deoxycholic acid in large bowel carcinogenesis. Nutr Cancer 1987; 9: 67-71
34. Paganelli GM, Biasco G, Brandi G, Santucci R, Gizzi G, Villani V, Cianci M, Miglioli M, Barbara L: Effect of vitamin A, C, and E supplementation on rectal cell proliferation in patients with colorectal adenomas. J Natl Cancer Inst (in press).
35. Schatzkin A, Freedman LS, Schiffman MH, Dawsey SM: Validation of intermediate end points in cancer research. J Natl Cancer Inst 1990; 82: 1746-1752