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Intermediate Biomarkers of Increased Risk for Colorectal Cancer: Comparison of Different Methods of Analysis and Modifications by Chemopreventive Interventions

Alessandra Scalmati and Martin Lipkin

Irving Weinstein Laboratory for Gastrointestinal Cancer Prevention, Gastroenterology Service and Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

Abstract Intermediate biomarkers of abnormal cell growth and development have recently been used in chemoprevention trials in attempts to identify the efficacy of chemopreventive agents in human subjects. Measurements carried out include those related to cell proliferation, differentiation, and gene structure and expression in the colon. Among modified patterns of cell proliferation identified by microautoradiographic or immunoperoxidase assays, a characteristic expansion in the size of the proliferative compartment has been observed in normal-appearing colorectal mucosa of human subjects with diseases increasing cancer risk; the same patterns have been induced by chemical carcinogens in rodents. Moreover, this intermediate biomarker has been modulated by chemopreventive agents in both rodents and humans. Newer intermediate biomarkers being studied for application to human chemoprevention programs include normal and abnormal patterns of expression of mucins, intermediate filaments and cytoskeletal proteins, and the structure and expression of a variety of genes associated with normal and abnormal cell development. The application of these various intermediate biomarkers to chemoprevention studies is increasing the ability of investigators to analyze the effects of novel chemopreventive agents in the colon and in other organs.

Key words: chemoprevention, colorectal cancer, differentiation, expansion of the proliferative compartment, intermediate biomarker, proliferation

INTRODUCTION

Colorectal cancer is a leading cause of morbidity and mortality in Western countries; its prognosis has been only slightly affected by improvements in chemotherapy and surgery [1]. Studying this disease offers advantages of analyzing the evolution of recognized precancerous lesions and new approaches to their prevention, the multistep evolution of human tumors, and important factors in the development of hereditary syndromes. These factors account for an expanded interest in colorectal cancer that includes new approaches to the chemoprevention of the disease [2].

In identifying diseases predisposing to colorectal cancer, and precancerous lesions developing during the evolution of the disease, subgroups of the population at increased risk have been defined as groups that might benefit from chemopreventive interventions. This has contributed to the further analyses of intermediate biomarkers of cell proliferation and differentiation in populations at increased risk, that can now be applied to studies of chemopreventive agents [3].

CLASSIC MEASUREMENTS OF CELL PROLIFERATION WITH [^{3}H]dThd

Among measurements that have been available for a long duration, the determination of cell proliferation by [^{3}H]dThd and microautoradiography has been widely used. [^{3}H]dThd is incorporated into newly formed DNA of cells in S-phase of the cell cycle, and the location of these cells in colonic crypts has been extensively investigated both in human subjects and in

rodents. With these measurements a spatial pattern of cell proliferation in colonic crypts typical of normal human subjects and rodents has been described, together with alterations occurring during disease development.

In low-risk mucosa the proliferative compartment is confined to the lower portion of colonic crypts. In normal appearing colorectal mucosa of patients with familial polyposis the total labelling index (ratio between labelled and total cells) characteristically is not increased; but when the distribution of labelled cells along the crypt is measured an abnormal pattern has been observed with two main alterations described—an expansion of the proliferative compartment toward the top of the crypt, and a shift of the entire proliferative compartment from the lower crypt to the lumenal surface [4–6]. These changes in cell proliferation have been found in normal mucosa of patients with sporadic adenomas and cancers, and in some instances an increase in total labelling index also has been observed [7–10].

In the flat mucosa of patients with long-standing ulcerative colitis an expansion in the size of the proliferative compartment has also been observed [11,12], as in patients with familial colon cancer [13], in aging [14], and in the remaining mucosa after subtotal colectomy for colorectal cancer [15]. The pattern of cell proliferation has shown either an expansion of the proliferative compartment toward the crypt surface, an overall hyperproliferation, or both.

The use of [^{3}H]dThd with microautoradiography for cell proliferation measurements has several advantages: it has been a well-standardized technique, reliable, quantifiable and reasonably inexpensive. However, it requires some excess time and it implies the use of a radioactive compound. Moreover, despite having better sensitivity and specificity for population groups, it has had less predictive value for individuals.

NEWER BIOMARKERS OF CELL PROLIFERATION

Measurements of cell proliferation have recently been carried out using bromodeoxyuridine (BrdU) incorporation into newly formed DNA, and immunoperoxidase detection of BrdU in tissue sections. BrdU is an analog of thymidine incorporated into newly replicating DNA of cells during S-phase of the proliferative cell cycle. Advantages are that it is not radioactive, and the technique is somewhat faster than autoradiographic measurements.

Proliferating cell nuclear antigen (PCNA) is also detected by immunohistochemistry. This cell cycle associated protein is expressed during a somewhat broader part of the cell cycle, and its measurements have corresponded fairly well to [^{3}H]dThd incorporation into normal cells [16], but its pattern of expression in diseased cells is still unclear. The same problem is true for Ki67, another cell cycle related antigen, which moreover has the disadvantage of requiring frozen tissue for determination.

A further approach has been taken with the biochemical analysis of polyamines and ornithine decarboxylase (ODC) [17]. Polyamine metabolism is linked to cell proliferation, and a key enzyme, ODC, has been shown to be increased in rapidly proliferating tissues. This technique presents basically two disadvantages: first the determination is influenced by tissue storage and other factors related to stability; a second problem with its use in colonic mucosa occurs because this marker can only detect changes in overall proliferation in the tissues, and can't be used to determine proliferative activity in different crypt compartments.

Using a cytofluorimeter it is possible not only to determine the DNA content of the cells, but also to measure cell proliferation in different cell cycle phases, so that the percent of cells in S-phase can be derived. A major problem with this technique is that it is not possible to maintain the histological structure of the tissue being examined.

In several types of malignant cells an increased number of nucleolar organizer regions (NORs) have been reported to be related to cell proliferation. AgNOR determinations have been investigated mainly in malignant tissues, and its evaluation in preneoplastic lesions is still in a preliminary phase and therefore not clear at present.

COMPARISON OF RESULTS OBTAINED WITH DIFFERENT BIOMARKERS

In order to interpret the results of biomarker measurements obtained in different laborato-

TABLE I. Comparison of Results Obtained With Different Biomarkers

Cell Type	Biomarkers	References
Large Intestine human	BrdU and [^{3}H]dThd	[20]
	BrdU and PCNA	[21]
	AgNOR, PCNA, Ki67, and DNA α polymerase	[22]
	AgNOR and PCNA	[23]
	Ki67 and ODC	[24]
	BrdU and ODC	[25]
	Amaranthin and BrdU	[26]
	p53 protein expression and Ki67	[27]
	p53 protein expression and PCNA	[28]

ries, and to begin to assess the value of newer and therefore less standardized techniques, comparison studies have been carried out. More standardizations have been carried out in animal models and fewer in human studies; Table 1 shows results of some studies carried out in humans.

Labelling indices (LI) obtained with [^{3}H]dThd and BrdU have been shown to be generally comparable [18,19], however not in all instances [20] with LI detected by BrdU lower than with [^{3}H]dThd. BrdU and PCNA have given comparable results in normal mucosa in a few studies, but the increase in PCNA-LI in adenomas with increasing dysplasia was greater than that of BrdU-LI, so that the correlation between the two markers was less in diseased tissue [21]. PCNA, Ki67, and AgNOR also gave comparable results in mild dysplastic adenomas, and their increases paralleled proliferation of the lesions toward microinvasive carcinomas [22]. A positive correlation was found between AgNOR and PCNA with an increase in both markers progressing from normal mucosa to mildly and severely dysplastic polyps [23].

No increase in proliferative activity in transitional mucosa (within 2 cm of a colorectal cancer) was found using either ODC or Ki67, and no expansion of the proliferative compartment was noted [24]. However, ODC activity and BrdU-LI both increased in normal mucosa of patients with adenomas [25]. Amaranthin is a lectin reported to label the proliferative compartment of colonic crypts including cells not in S-phase, and a general correlation between this new marker and LI measured by BrdU was found [26].

p53 is believed to have a tumor suppressor function located on chromosome 17, and can be either mutated or deleted in colorectal cancer. The nuclear phosphoprotein gene product may be involved in control of normal cell growth. The level of p53 protein is undetectable by immunohistochemistry in normal mucosa, but it can stain positively in adenomas and adenocarcinomas. Relationships between p53 protein

TABLE II. Effects of Chemopreventive Agents on Biomarkers of Increased Susceptibility to Colorectal Cancer

Agent	Effect	References
Calcium	Decreased hyperproliferation	[29]
	Decreased hyperproliferation	[30]
	Decreased hyperproliferation	[31]
	Unchanged hypoproliferation	[32]
	Decreased ODC activity in patients with polyps	[33]
Fiber	Wheat bran (13.5 gr. fiber) decreased rectal cell proliferation	[35]
	Wheat bran reduced polyps	[36]
Vitamins	Beta-carotene 20 mg daily for 2 years contracted the zone of cell proliferation toward the crypt base in patients with polyps	[41]
	Beta-carotene 30 mg daily for 6 months decreased ODC in normal rectal mucosa of patients with history of colon cancer	[40]
	Vitamin A (30,000 U), Vitamin C (1 g) and Vitamin E (70 mg) daily for 6 months reduced the size of the proliferative compartment in flat rectal mucosa of patients with adenomas	[37]
	Ascorbic acid 3 g daily for 18 months decreased cell proliferation and number of polyps in FAP patients	[39]
	Vitamin A, C and E daily for 2 years reduced the recurrence of colorectal adenomas	[38]
	Beta-carotene 20 mg daily for 2 years did not decrease the recurrence of adenomatous and hyperplastic polyps	[42]
ω3 FA	ω3 fatty acids reduced the size of the proliferative compartment in flat mucosa of patients with colorectal adenomas	[44]

expression and cell proliferation have been studied, with lack of correlation using Ki67 as a marker of proliferation [27], and increased expression of p53 in highly proliferative lesions using PCNA as a marker of cell proliferation [28].

These examples of lack of correlation of proliferative indices using sets of relatively new biomarkers clearly show the importance of carrying out comparison studies. Combinations of complementary biomarkers may have advantages in defining different cell functions when carefully chosen and standardized. Some of the results thus far obtained are still inconsistent and the functions of some biomarkers within cells are not clear.

EFFECT OF CHEMOPREVENTIVE AGENTS ON INTERMEDIATE BIOMARKERS

Table 2 shows early results of clinical studies in which the expressions of intermediate biomarkers were modified by chemopreventive agents. The results obtained in rodent models and in most human studies using calcium have been fairly consistent. Supplementary dietary calcium (1250–2000 mg daily) has decreased hyperproliferation of colonic epithelial cells in rodents and in patients at increased risk for colon cancer [29–31], but calcium has not decreased already low levels of cell proliferation [32]. Supplemental calcium also has decreased ODC activity in patients with polyps [33].

The results obtained by increasing fiber in the diet in rodent models have differed depending on the type and amount of fiber; epidemiologic studies suggest that a human diet high in fiber has a protective role against neoplasia [34]. The studies enumerated in Table 2 show decreased cell proliferation and polyp recurrence with increased fiber, and further studies are needed to confirm the effects of fiber in humans [35,36].

Effects of vitamins on cell proliferation and polyp recurrence also have begun to be investigated. In studies utilizing high amounts of vitamins A, C and E both decreased cell proliferation and polyp recurrence were found [37,38]. Similar findings were noted with vitamin C [39]. β-carotene decreased ODC activity [40], contracted the size of the proliferative zone [41], and did not reduce polyp recurrence rate within two years [42]. A chemopreventive effect of $\omega 3$ fatty acids has been widely investigated in animals [43], and activity in humans has also been noted in reducing the size of the proliferative compartment [44].

CONCLUSIONS

Current findings have shown an increased application of intermediate biomarkers of cell proliferation, differentiation and gene expression in risk analysis and chemoprevention studies. During the early application of these biomarkers in order to avoid misinterpretation of results, standardization of newer biomarkers is essential. The application of newer biomarkers is likely to be very attractive and it can be very tempting to utilize newer and faster techniques; however, reproducibility of measurements in normal cells and in diseased cells may differ and should not be overlooked. These principles should be kept in mind in the development of this new area of human clinical investigation.

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