Genetic and Cellular Changes in Colorectal Cancer: Proposed Targets of Chemopreventive Agents

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Abstract

Progress in development of a genetic model for colorectal tumorigenesis and human chemoprevention research may allow the mechanism-based identification of targets and chemopreventive agents that will protect against colorectal cancer. For example, numerous mutagenic events can occur throughout colorectal carcinogenesis, including loss of heterozygosity in tumor suppressor genes such as APC, MCC, DCC, and p53, as well as in oncogenes such as K-ras. Chemopreventive agents that inhibit mutagenic activity such as N-acetyl-L-cysteine, oltipraz, and nonsteroidal anti-inflammatory drugs may protect against these mutations. Also, agents such as perillyl alcohol and lovastatin that interfere with protein isoprenylation and, hence, inhibit oncoprotein activation may protect against aberrant K-ras expression. Hyperproliferation in normal mucosa, leading to early adenomas, and cellular proliferation, leading to growth and progression of neoplasia, are also aspects of colorectal carcinogenesis that can be controlled by chemopreventive agents. Calcium is a chemopreventive agent for which there is both clinical and experimental evidence of inhibition of cell proliferation in colon mucosa. Other examples of antiproliferative agents with potential chemopreventive efficacy in colon are 2-difluoromethylornithine, dehydroepiandrosterone, and selenium. Differentiating agents such as retinoids and deltanoids may also slow proliferation and progression. Antioxidants have potential for interfering with both mutagenicity and proliferation (e.g., by preventing oxidative activation of carcinogens and scavenging activated oxygen species generated during inflammation). The same mechanistic principles apply to identification of dietary chemopreventive intervention for colorectal carcinogenesis. For example, lowering dietary fat and increasing dietary fiber lead to lower colorectal mucosal proliferation, and cruciferous vegetables contain agents such as indoles and dithiolthiones that have shown antimutagenic activity.

Introduction

Rapidly evolving progress in two very important areas, development of a genetic model for colorectal tumorigenesis and human chemoprevention research, have brought about new possibilities for innovative approaches to the prevention and control of colorectal cancer. Research advances in cell genetics are providing approaches for the identification of individuals at high risk for colorectal cancer. For example, the hMLH1 gene on chromosome 3p and the hMSH2 gene on chromosome 2p have recently been linked to hereditary nonpolyposis colon cancer. hMLH1 and hMSH2 are thought to play a central role in the DNA repair pathway, and mutations in these genes result in an accumulation of genetic errors that may increase susceptibility to hereditary nonpolyposis colon cancer (1, 2). The ability to identify individuals susceptible to colorectal cancer will put forth the challenge of reducing the risk of tumor development for these individuals. Chemoprevention, an area of cancer prevention research that recognizes carcinogenesis to be an evolving multistep molecular and cellular process, may be a way to reduce this risk. In chemoprevention, noncytotoxic nutrients or pharmacological compounds that protect against the development and progression of mutant clones of malignant cells are used to either inhibit or reverse carcinogenesis (3, 4).

Clearly, an understanding of the mechanisms that trigger and drive the carcinogenic process is critical to achieving the goal of cancer prevention via specific chemopreventive agents. For some types of cancer (e.g., colorectal cancer), the disease appears to result from genetic mutations, mostly acquired, that accumulate in the genome of the evolving cancer cell (5–8). Vogelstein et al. (5), Fearon and Vogelstein (6), and Fearon and Jones (9) have developed a preliminary genetic model for colorectal tumorigenesis that indicates that several genetic mutations are required for malignant tumor development, with the order of occurrence of the genetic mutations being less important than the number of mutations. The molecular events that bring about such genetic mutations are possible targets for chemoprevention of neoplastic progression in colorectal cancer.

Although the development of chemopreventive agents has evolved largely from lines of research other than cell genetics, examination of mechanisms of action of candidate chemopreventive agents for colorectal cancer suggest that these agents should be beneficial in preventing or treating mutations associated with colorectal tumorigenesis. For example, direct evidence for a mechanism by which NSAIDs such as aspirin,

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indomethacin, ibuprofen, piroxicam, and sulindac may inhibit colon carcinogenesis is lacking; however, it has been established that NSAIDs interfere with PGH synthesis from arachidonic acid by inhibiting the cyclooxygenase activity of PGH synthase (10). Such inhibition interferes with the PGH synthase-catalyzed oxidation of chemical carcinogens, such as aromatic and heterocyclic amines, to mutagenic derivatives that may play a role in colorectal carcinogenesis (10–12). Inhibition of cyclooxygenase activity also reduces the endogenous formation of MDA in platelets: MDA has been shown to be mutagenic in bacterial and mammalian test systems and carcinogenic in rats (10). Mutations induced by MDA include the base pair C→T transitions frequently associated with mutations in the p53 gene in the human colon (13). As another example, the chemopreventive agent oltipraz may block or suppress mutations by enhancing carcinogen detoxification through induction of xenobiotic-metabolizing enzymes (14, 15). Further, folic acid and SAM are methylating agents that have the potential to prevent hypomethylation of DNA, which is observed in colorectal neoplasia in humans and may contribute to the loss of normal controls on proto-oncogene expression (6–19).

Much research remains to be carried out to elucidate the mechanisms of action of potential chemopreventive agents. Fig. 1 illustrates the progression of histopathological changes [from normal epithelium to hyperproliferative changes to intraepithelial neoplasia/dysplasia (adenoma) to invasive neoplasia (carcinoma and metastasis)] as well as the probable genetic and molecular events associated with these changes. The figure also outlines the classes of chemopreventive agents that have the potential for reducing colorectal cancer risk and indicates which genetic, molecular, and histopathological changes these agents might affect. The remainder of this paper briefly describes the colorectal tumorigenesis model and discusses the possible relationships between potential chemopreventive agents and the changes reflected in this model.

The Colorectal Tumorigenesis Model

As illustrated in Fig. 1, colorectal tumorigenesis is considered to be a complex multistep process in which cells accumulate multiple alterations of genes that control cell growth and differentiation, resulting in a neoplastic phenotype (6, 9, 20–22). Intraepithelial neoplasia, which includes the appearance of morphologically altered cells observed as dysplasia (as in adenomas), occurs before invasion across the basement membrane. Intraepithelial neoplasia is characterized by pathologists as benign but as having a greatly increased degree of risk for progression to the invasive stage, at which point it is termed malignant, carcinoma, or cancer (23, 24).

As described by Fearon and Vogelstein (6), the combined effects of the mutational activation of oncogenes, the activity of which leads to enhanced cell growth, and the inactivation of several tumor suppressor genes, negative regulators of cellular proliferation, play a significant role in colorectal tumor development (20–22). When tumor suppressor genes are inactivated by either inherited or somatic mutations, a block to cellular proliferation is removed and cells begin deregulated growth that may lead to tumor formation (9, 21). Overall, alterations in at least four to five genes are required for the formation of a malignant tumor (6). Although genetic alterations appear to occur at specific stages during colorectal tumor progression, it is the total accumulation of alterations, rather than their order with respect to one another, that is most important in determining the neoplastic phenotype (6, 8, 9, 20, 25).

The series of genetic alterations involved in colorectal tumorigenesis identified thus far include the K-ras oncogene on the short arm of chromosome 12 and several tumor suppressor genes, including the adenomatous polyposis coli (APC) and “mutated in colon cancer” (MCC) genes on chromosome 5q, DCC gene on chromosome 18q, and the p53 gene at chromosome 17q (6, 26). Because colorectal tumors evolve through well defined morphological stages, it has been possible to approximate the order in which these genetic alterations occur. Mutations at the APC tumor suppressor gene on chromosome 5q appear early in the development of some colorectal tumors (27, 28). Early adenoma is believed to arise from hyperproliferative epithelial cells as a result of this mutation. In patients with FAP (a syndrome characterized by the progressive growth of hundreds of adenomatous colorectal polyps, some of which progress to cancer), a mutation on chromosome 5q is inherited. In tumors arising in patients without polyposis, the same region may be lost or mutated (6, 29, 30). MCC, the tumor suppressor gene that has been identified on chromosome 5q, is somatically altered in sporadic colorectal cancer (17, 27).

Another relatively early event in colorectal tumorigenesis is a significant loss of methyl groups in DNA. Aberrant DNA methylation is evident even in small tubular adenomas (31). Hypermethylation may contribute to instability in the tumor cell genome and thus may affect the rate at which genetic alterations, such as allelic losses, occur (6). The K-ras oncogene mutation on the short arm of chromosome 12 also is an important early somatic alteration, as evidenced by its high frequency of occurrence in adenomas (32, 33). It has been suggested that, through clonal expansion, K-ras mutation and DNA hypomethylation lead to larger and more dysplastic adenomas (6, 28).

Deletion of the DCC gene on chromosome 18q, as well as mutation and deletion of the p53 gene on chromosome 17p, appear to be relatively late events in colorectal tumorigenesis, with high prevalence only in later stage adenomas and in colorectal carcinomas (9, 28, 31, 34). These deletions are associated with the transition from preinvasive neoplasia to invasive (malignant) carcinoma. The accumulated loss of suppressor genes on additional chromosomes appears to correlate with the ability of invasive carcinoma to metastasize (6).

In general, the progression of neoplastic events in tumorigenesis gives rise to genetic, biochemical, and biological factors that can be used to monitor the stages of this process (24). These biomarkers can serve as surrogate or intermediate end points for cancer in clinical trials of chemopreventive agents; that is, they represent changes in the continuum of events between the initiation of carcinogenesis and the final expression of clinically evident disease (24, 35). The specific morphological features that characterize intraepithelial neoplasia (i.e., nuclear area, shape, texture, and the increased variation of these features) can themselves serve as reliable SEBs that can be assayed to evaluate modulation by chemopreventive agents (24, 36, 37). For example, the regression of colorectal adenomatous polyps, which have a certain probability of progressing to invasive neoplasia, can be used as a SEB for inhibition of colorectal cancer. Sulindac, a NSAID, has been shown to cause significant regression of polyps in FAP patients and may therefore reduce the risk of subsequent colorectal cancer development in these patients (24, 38).

In addition to characteristic morphological changes, the development of intraepithelial neoplasia is associated with
the appearance of a number of other individual biomarkers characterized as genomic (e.g., oncogene activation, gene amplification), proliferative (e.g., thymidine labeling index, bromodeoxyuridine uptake, and proliferating cell nuclear antigen), and differentiation related (e.g., abnormal glycoconjugate antigens; Refs. 23, 24). Biomarkers that correlate closely to the presence and progression of intraepithelial neoplasia in colorectal cancer will facilitate the evaluation of chemopreventive agents that have the potential to inhibit or reverse intraepithelial neoplasia in colorectal tumorigenesis. For example, a short-term Phase II clinical trial of calcium as a chemopreventive agent for colon cancer is evaluating the use of thymidine labeling index, bromodeoxyuridine uptake, extended Lewis x antigen, keratins, and integrins as molecular SEBs, as well as ploidy, nuclear morphometry, and nuclear texture as morphological SEBs (24). SEBs that can accurately indicate a specific stage of tumorigenesis provide targets for designing logical and potentially effective chemopreventive interventions. Such SEBs are needed to supplant the end point of cancer incidence reduction, which requires large study populations, long observation periods, and great expense (23, 39).

Chemopreventive Agents in Relation to the Colorectal Tumorigenesis Model

**Chemopreventive Mechanism Classification**

In general, chemopreventive agents can be grouped into three broad major classes: carcinogen-blocking agents, agents that suppress promotion, and antioxidants (36, 40, 41). Blocking agents (such as oltipraz, NAC, and ellagic acid) prevent carcinogenic compounds from reaching or reacting with critical target sites by inhibiting the metabolic activation of carcinogens that is catalyzed by cytochrome P450 (Phase I enzymes); enhancing detoxification systems (e.g., Phase II enzymes such as GSH S-transferase, involved in conjugation and excretion reactions); and trapping reactive carcinogens before they reach critical target sites by inhibiting the metabolic activation of carcinogens that is catalyzed by cytochrome P450 (Phase I enzymes); enhancing detoxification systems (e.g., Phase II enzymes such as GSH S-transferase, involved in conjugation and excretion reactions); and trapping reactive carcinogens before they reach critical target sites (40, 42). Agents that suppress promotion prevent evolution of the neoplastic process in cells that would otherwise become malignant (40, 41, 43). Although the mechanisms of action of suppressing agents have not been clearly defined, research indicates that these agents include differentiating agents (e.g., retinoids, deltoids), inhibitors of oncogene action (e.g., terpenes), selective inhibitors of cell proliferation (e.g., DFMO, calcium), and anti-inflammatory agents, such as...
NSAIDs (36, 40). Antioxidants such as β-carotene, vitamin E, and curcumin scavenge oxygen free radicals and organic free radicals and terminate lipid peroxidation, either directly or indirectly. Antioxidants with indirect effects are exemplified by those that enhance Phase II metabolizing enzymes, resulting in elevated electrophile trapping potential via increased GSH production and induction of GSH peroxidase (36, 40). A chemopreventive agent such as d-limonene, which is a blocking agent, an antiproliferative (e.g., ras prenylation inhibitor), and an antioxidant, has the potential to target multiple genetic or cellular events in the transformation of normal cells to cancer cells as illustrated in the colorectal tumorigenesis model (36). Combinations of agents can increase efficacy; for example, synergistic chemopreventive activity has been reported for DFMO and piroxicam in colon cancer in rats (44). Reduced toxicity can be a benefit of sequential or simultaneous administration of chemopreventive agents because dose levels can be reduced when efficacy increases.

In Fig. 1, the chemopreventive agents that have either demonstrated a beneficial effect or have potential for preventing colorectal cancer are classified as agents that block or suppress mutation, suppress proliferation, act as differentiating agents, and prevent hypomethylation. The remainder of this section discusses both the recognized and proposed actions of these chemopreventive agents at various stages in the histopathology of colorectal cancer.

**Genetic Changes as Targets of Chemopreventive Agents**

As described for the colorectal tumorigenesis model, point mutations in the ras genes and allelic deletions in chromosomes 5q, 17p, and 18q are the most common of the genetic alterations observed in colorectal cancer, and data suggest that the accumulation rather than the sequence of these alterations appears to be more influential in regulating cell growth, proliferation, and differentiation (5, 6, 9, 45, 46). Thus, use of chemopreventive agents that have potential to prevent gene mutations that contribute to colorectal carcinogenesis is a rational approach to the prevention of colorectal cancer.

**Agents That Block/Suppress Mutations.** All carcinogens that could cause mutations in the APC, MCC, DCC, and p53 genes (tumor suppressor genes), MMLH1 and hMSH2 genes (mutator genes), and the K-ras oncogene are potential targets for chemopreventive agents. Further, there are undoubtedly additional tumor suppressor genes and oncogenes involved in colorectal tumor development that remain to be identified (9), for which chemopreventive agents might be beneficial in terms of preventing somatic mutations. Numerous classes of chemicals, many naturally occurring, such as flavonoids, indoles, isothiocyanates, and dithiolthiones, can block carcinogen activation by inducing anticarcinogenic enzymes (47, 48). Anticarcinogenic enzyme inducers can be either monofunctional inducers, which elevate both the Phase I enzymes that catalyze the metabolic activation of carcinogens (e.g., cytochrome P450) and Phase II enzymes (42, 48, 49). The monofunctional Phase II enzyme inducer (e.g., dithiolthione) appears to be a more promising chemopreventive agent. By measuring the effect of various compounds on the activity of an indicator enzyme, NADPH:quinone oxidoreductase, in murine hepatoma cells that exhibit Phase II but not Phase I enzymatic function, Talalay et al. (49) determined that Phase II enzyme inducers are Michael reaction acceptors that are electrophilic due to conjugation with electron-withdrawing substituents and, thus, are susceptible to attack by nucleophiles. Further, the potency of the inducers parallels their efficiency in Michael reactions, providing a link between the structure of Phase II enzyme inducers and potential chemopreventive activity (49). The practical importance of this finding is exemplified by the later development of a simple, rapid colorimetric assay for detecting compounds that selectively induce Phase II enzymes (also based on the activity of NADPH:quinone oxidoreductase in murine hepatoma cells). This assay was used to screen vegetable extracts for NADPH:quinone oxidoreductase activity (50) and, consequently, to isolate and identify sulforaphane as a potent monofunctional Phase II enzyme inducer in broccoli (51).

Chemopreventive agents currently under investigation that either block or suppress mutation and that show promise for reducing colorectal cancer risk include S-allyl-L-cysteine, NAC, oltipraz, and DHEA, as well as d-limonene and its hydroxylated derivative, perillyl alcohol. It is postulated that these agents have the potential to prevent genetic mutations that occur at any stage in colorectal tumorigenesis. S-allyl-L-cysteine, NAC, and oltipraz, all organosulfur compounds, are Phase II metabolic enzyme inducers and electron scavengers (15, 36, 52). S-allyl-L-cysteine, a naturally occurring compound in garlic stem, appears to block the oxidation of DMH and inhibits DMH-induced tumors in mice (53, 54). NAC, which shows chemopreventive activity in rat and mouse colon models (36, 52, 55, 56), increases intracellular GSH levels and increases the activity of GSH S-transferases. This agent enhances detoxification of various carcinogens (e.g., epichlorhydrin, 4-nitroquinoline-N-oxide, aflatoxin B1, benzo[a]pyrene; Ref. 57). Oltipraz, a synthetic dithiolthione related to naturally occurring dithiolthiones found in cruciferous vegetables, has demonstrated significant chemopreventive efficacy in both mouse and rat colon models in screens carried out under the NCI chemopreventive drug development program (36, 58, 59). Oltipraz induces Phase II enzymes (e.g., GSH S-transferases and epoxide hydrolase; Refs. 15, 59, 60).

DHEA is a weakly androgenic and weakly estrogenic adrenal corticoid steroid that is found in human serum and is a normal tissue precursor for androgens and estrogens (61, 62). Although the chemopreventive activity of DHEA has been demonstrated for carcinogen-induced colon, lung, mammary, and skin tumors in animal models (61–64), the mechanisms by which DHEA may modulate carcinogenesis are not clearly understood. It is recognized, however, that DHEA is a potent inhibitor of glucose-6-phosphate dehydrogenase, which results in inhibition of the pentose phosphate pathway. This inhibition results in decreased availability of cellular NADPH that is required for cytochrome P450 activation of carcinogens and, consequently, may block this activation and resulting mutations (62, 65). Because DHEA has some undesirable androgenic effects, several analogues have been designed with lesser side effects. Fluasterone (16α-fluoro-DHEA), which is nonandrogenic, is particularly promising and is being developed by the NCI chemopreventive drug program (36).

d-Limonene, a plant monoterpene and major constituent of citrus fruit oils, inhibits carcinogenesis when administered shortly before carcinogen exposure (41). Although no data are available for the effect of d-limonene on colorectal cancer, the compound is reported to be an effective inhibitor of the initiation and promotion stages of mammary carcinogenesis at high doses (66, 67). Further, d-limonene selectively inhibits isoprenylation of proteins, including the G protein members of the p21⁰⁰ family (66, 68), affecting ras activity and ras-driven cellular proliferation; these effects on mutated K-ras expression...
are presented in more detail below in the discussion of agents that suppress promotion.

**Antioxidants.** By virtue of their ability to trap electrophilic sites on activated carcinogens, scavenge free radicals, terminate lipid peroxidation, and enhance electrophile trapping potential by inducing Phase II metabolizing enzymes, antioxidants have the potential to inhibit mutations in the genes currently known to be associated with colorectal tumorigenesis. Antioxidants that have the potential to reduce cancer risk by targeting the genetic changes associated with colorectal tumorigenesis include the NSAIDs, polyphenols, vitamin E, curcumin, fumaric acid, genistein, and quercetin.

NSAIDs are anti-inflammatory agents by inhibiting the synthesis of PGHs and other eicosanoids through inhibiting the cyclooxygenase and hydroperoxidase activities of PGH synthase and lipooxygenase, thereby interfering with the arachidonic acid metabolism cascade (10). Inhibition of PGH synthesis leads to reduced cell proliferation, probably as a result of multiple mechanisms including inhibition of activated oxygen radicals formed during inflammation (10). However, as antioxidants, NSAIDs may interfere with carcinogenesis by inhibiting the co-oxidation of proximate carcinogens (36, 40, 69). In addition to the NSAIDs, many naturally occurring anti-inflammatory agents exist that are potent antioxidants, including phenolic and polyphenolic compounds such as flavonoids, catechins, and curcumin. For example, epigallocatechin gallate, a polyphenol that occurs in tea, has been shown to have anti-inflammatory properties and to be a potent antioxidant (40).

Naturally occurring polyphenols have received considerable attention as potential chemopreventive agents. The polyphenols from green (e.g., catechins) and black (e.g., theaflavins, thearubigens) tea extracts inhibit the chemical induction of tumors at multiple sites in rodents. Their principal action as antioxidants is to protect against oxidative damage, including lipid peroxidation and DNA strand breaks (70–72). Ellagic acid, a naturally occurring polyphenol that is found in numerous fruits and vegetables, has been shown to reduce tumor incidence in rat small intestine when fed in the diet (73). In addition to its antioxidant potential, the chemopreventive activity of ellagic acid appears to be related to its ability to prevent metabolic activation of carcinogens and binding of the activated carcinogens to DNA (74–76).

Vitamin E (α-tocopherol), the major lipid-soluble antioxidant found in cell membranes, inactivates free radicals and reactive oxygen species to prevent oxidative damage to membrane lipids, protein denaturation, and nucleic acid alterations; thus, vitamin E has the potential to protect colon epithelial cells against DNA damage (77–80). Overall, animal and epidemiological studies on vitamin E and cancer prevention have yielded mixed results (79, 81). However, results of a prospective cohort study of more than 32,000 Iowa women indicate that a high intake of vitamin E is associated with a significant decreased risk of colon cancer, especially in persons under age 65 (82). The relative risk for the highest quintile of total vitamin E intake (dietary vitamin E plus supplements) compared with the lowest quintile was 0.42, after multivariate adjustment for age and other dietary antioxidants. This protective effect of vitamin E, however, was age dependent; no significant beneficial effect was observed for women older than 65, suggesting that risk of colon cancer may be influenced by an interaction between vitamin E intake and age. In an intervention study, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (conducted in Finland in more than 29,000 male cigarette smokers ages 50–69 years), a daily supplement of vitamin E reduced colorectal cancer incidence by 16% and prostate cancer incidence by 34% (83).

Curcumin, the yellow pigment in turmeric, exhibits strong antioxidant activity and is an effective scavenger of superoxide radicals. It may also affect the metabolic activation and DNA binding of polynuclear aromatic hydrocarbons (84). A potent anti-inflammatory agent as well, it reportedly inhibits arachidonic acid metabolism in mouse skin, blocking both the lipooxygenase and cyclooxygenase pathways (84). As part of the NCI chemopreventive drug development program, this compound showed chemopreventive efficacy in a MAM-induced mouse colon cancer model (36, 85). It also inhibited AOM-induced colon tumor incidence, multiplicity, and volume in male F344 rats (86).

The mechanism of the chemopreventive activity of fumaric acid has not been elucidated but may be related to its antioxidant potential. Fumaric acid appears to be active in later stages of carcinogenesis; for example, it inhibited tumor development in mouse forestomach, mouse lung, and rat liver when administered after treatment with a carcinogen (36). Also, fumaric acid inhibited the AOM-induced production of aberrant crypt foci in rats (85).

**In vivo** antioxidant activity reported for the isoflavone genistein includes inhibition of lipooxygenase and inhibition of a tyrosine protein kinase required to stimulate phospholipase C, resulting in the inhibition of phospholipase C-associated oxidative stress (87). Further, the isoflavone genistein has been shown to inhibit the activity of DNA topoisomerase II, a nuclear enzyme that catalyzes the formation of single- or double-strand DNA breaks (88). In addition to its antioxidant activity, genistein is known to inhibit the intrinsic tyrosine protein kinase activity of many growth factor receptors, including epidermal growth factor receptor and platelet-derived growth factor receptor (88–91). A recent report indicates that genistein inhibits angiogenesis (blood vessel proliferation required for the growth and metastasis of tumors) by inhibiting the effects of basic fibroblast growth factor on endothelial cell proliferation through its activity as a tyrosine kinase inhibitor (92). Thus, genistein could have a role in inhibiting progression to disseminated malignancy (93, 94). Further, this isoflavone can induce cytostatic effects on cell growth independent of tyrosine protein kinase activity, possibly through its inhibition of ribosomal S6 phosphorylation (93).

**Agents That Prevent Hypomethylation.** Both folic acid and SAM are chemopreventive agents that may have potential for preventing hypomethylation, thereby reducing colorectal cancer risk. The hypomethylation of DNA that occurs in adenomatous polyposis precursors and in colorectal cancers is associated with low levels of SAM, which is required for DNA methylation. Both low dietary folate and methionine may reduce methyl group availability and, consequently, reduce levels of SAM (93). DNA hypomethylation is an early step in colorectal carcinogenesis (45, 95). Aberrant patterns of hypomethylation may lead to changes in DNA conformation that alter DNA-protein binding patterns and contribute to genetic instability (96), resulting in the loss of normal controls on proto-oncogene expression (16, 17). Thus, hypomethylation of a nonexpressed gene (e.g., ras oncogene) might result in expression of that gene, contributing to the development of tumorigenesis (97). Analysis of methylation patterns of oncogenes in DNA from colorectal cancer patients reveals substantial alterations of methylation patterns in the majority of tumors (97, 98), with c-H-ras being hypomethylated more frequently than c-K-ras in cancer tissue (32, 33). Further, methyl-deficient diets in animals...
have been observed to lead to hypomethylation of ras onco-
genomes in both hepatomas and colon carcinomas (97, 99). Overall, existing evidence provides a rational basis for the concept that folic acid deficiency may be a factor in cancer initiation. In fact, assessment of dietary intake for a 1-year period for women in the Nurses’ Health Study and for men in the Physicians’ Follow-Up Study found that high dietary folate was inversely associated with risk of colorectal adenoma in women (relative risk = 0.66) and in men (relative risk = 0.63) after adjusting for age, family history, indications for endoscopy, history of previous endoscopy, total energy intake, saturated fat intake, dietary fiber, and body mass index (94).

**Cellular Changes as Targets of Chemopreventive Agents**

The evaluation of colorectal tumors from individuals, both with and without FAP, has helped to characterize the cellular alterations associated with cell proliferation. Among the earliest changes in the progression from normal epithelium to colorectal carcinoma is the focal growth of normal-appearing, but disorganized, cells. An example of this state is the “aberrant crypt” that appears in the colon of rats after administration of AOM (24, 100). The individual cells in aberrant crypts are normal in appearance but form a disorganized and distorted crypt-like structure that tends to compress adjacent normal cells. Later, dysplastic cells appear, and the crypts are at high risk for developing into adenomas (23, 24, 100). Beginning with hyperproliferative changes and microadenoma, cellular changes (termed intraepithelial neoplasia) progress through early, intermediate, and late adenoma. When neoplastic epithelial cells of the adenoma exhibit signs of invading across the basement membrane and the adjacent muscularis mucosa (101), the condition is termed carcinoma (9, 23, 24). Chemopreventive agents that show promise for inhibiting the histopathological changes associated with colorectal tumorigenesis include agents that suppress promotion such as calcium, DFMO, selenium compounds, DHEA, NSAIDs, lovastatin, and 4-hydroxynonenen; differentiating agents such as the retinoids and diltiazem; and the antioxidants described above.

**Agents That Suppress Promotion.** In humans, calcium intake has been correlated to a significant decrease in colon cancer risk and a significant decrease in cell proliferation in colonic mucosa (102–104). One proposed explanation for the inhibition of colorectal carcinogenesis by dietary calcium is that, by forming insoluble complexes, calcium inactivates the bile acids and free fatty acids that are cytotoxic to colonic mucosal cells, resulting in inhibition of the homeostatic feedback mechanism that stimulates cell proliferation (105). Further, calcium supplementation in humans has been shown to reduce markedly the production of fecal DAG, a stimulator of cell proliferation, and to accelerate bacterial metabolism of DAG and its precursors (102). The potential relationship between DAG and cell proliferation is discussed in more detail below in “Dietary Fat.”

It is possible that calcium may have more direct anticarcinogenic effects. For example, in an experimental animal model using DMH-induced colonic tumors, calcium supplementation decreased the number of rats with multiple tumors, the tumor size, and the ratio of G-to-A K-ras gene mutations in the tumors, leading to the speculation that alterations in K-ras mutations may be a possible mechanism by which calcium influences colon carcinogenesis in this model (106).

DFMO is a potent, irreversible inhibitor of ODC. ODC catalyzes the conversion of ornithine to putrescine, the first and rate-limiting step in the synthesis of mammalian polyamines, which is closely linked to cell proliferation (36, 107). High levels of polyamines and polyamine synthetic enzymes such as ODC are found in colon tumors and other proliferating mucosal cells (108). Inhibition of polyamine synthesis in such cells may inhibit proliferation (23, 36, 44). DFMO has demonstrated chemopreventive activity in rat colon and is part of the NCI chemoprevention drug development program (36, 44, 73).

Selenium compounds inhibit carcinogen activation and decrease DNA binding of carcinogens; inhibit protein kinases; inhibit cell growth; exhibit anti-inflammatory activity; and may enhance GSH peroxidase, which catalyzes the reduction of H2O2 and organic peroxides, agents that cause oxidative damage to the cell (109–115). Collectively, these altered cellular activities may inhibit the development of early to late stage adenomas. Studies have shown that selenium compounds at nontoxic levels can inhibit cell growth (110, 116); chemopreventive effects have been demonstrated in experimental tumor models, including colon cancer in rats. Particularly, the aryalkyl selenium compounds, benzylselenocyanate (117) and p-xylylselenocyanate (118), have inhibited AOM-induced tumors in rat colon. Although the mechanism of the antiproliferative activity of selenium compounds has not been elucidated, inhibition of DNA (119), RNA (120), and protein synthesis (121) have been observed with selenium compounds, and all have been proposed to account for the antiproliferative activity of selenium. Selenobetaine and selenobetaine methyl ester, which are methylated organic selenium compounds, have been shown to reduce the long-term growth potential of a mouse leukemia cell line without inducing DNA strand breaks (i.e., are not cytotoxic; Ref. 116).

DHEA and its nonandrogenic analogues, such as flutasterone, may inhibit tumor promotion and antiproliferative activity (61–63). In addition to decreasing the availability of cellular NADPH, as noted earlier, inhibition of the pentose phosphate pathway by DHEA restricts the supply of five-carbon sugars required for synthesis of ribonucleosides and deoxyribonucleosides. Thus, DHEA may reduce the rate of nucleic acid synthesis, resulting in antiproliferative effects (62, 65). This is possibly supported by data that demonstrate that DHEA-induced growth inhibition, both in vitro and in vivo, can be reversed by introducing exogenous mixtures of ribonucleosides and deoxyribonucleosides into the model systems (61, 62, 65).

NSAIDs (aspirin, sulindac, ibuprofen, and piroxicam) all appear to suppress promotion, targeting cellular processes that affect early, intermediate, and late stages of adenoma development. As described above, these compounds inhibit components of the arachidonic acid cascade associated with inflammation (10, 40). Although PGH synthesis is thought to be essential for tumor promotion, and possibly metastasis, the specific mechanisms of PGH involvement are not yet clear (10). NSAIDs have demonstrated chemopreventive activity in both rat and mouse colon (36, 44, 122).

Several epidemiological studies suggest that aspirin inhibits the growth of colon polyps and lowers colon cancer risk (123–125). Results of a large prospective mortality study indicated that regular aspirin use (≥16 times/month) reduced the relative risk of death from colon cancer in adult men and women by 40–50% (124), but early evidence from the Physicians’ Health Study, in which more than 22,000 physicians were randomized to receive either aspirin or placebo for 5 years, was unable to demonstrate this effect (126). In preliminary clinical studies, sulindac caused almost total regression of adenomatous polyps in persons with Gardner’s syndrome and FAP (38, 127, 128). A goal of current chemoprevention re-
search is to determine dosages and regimens for NSAIDs that are efficacious while minimizing any adverse effects (36).

Lovastatin and d-limonene suppress promotion by inhibiting ras expression. Lovastatin, a drug currently used to treat hypercholesterolemia, blocks hydroxymethylglutaryl-CoA reductase, which is an early step in the sterol synthesis pathway. This ultimately leads to inhibition of isoprenylation, that is, the attachment of a farnesyl group, to p21ras proteins, the proteins produced by the ras oncogene. This protein modification is necessary for binding p21ras proteins and other proteins to cell membranes (93). D-limonene blocks isoprenylation by direct inhibition of farnesyl palmitoyltransferase (93, 129). Both compounds currently are being evaluated as potential chemopreventive agents for prostate and breast cancer. However, they could have significant benefits for other cancers, including colorectal cancer, that are associated with ras mutation and overexpression (93).

Differentiating Agents. Both the retinoids [vitamin A (retinol) and its synthetic analogues; Ref. 130] and the delta-noids [vitamin D, and its synthetic analogues; Ref. 131] are differentiating agents that inhibit proliferation and may have beneficial effects on intermediate and late stage adenomas.

Retinoids. The most studied chemopreventive retinoid, 13-cis-retinoic acid, reduces the risk of squamous cell carcinoma and oral leukoplasia; has been clinically shown to prevent second primary tumors after initial treatment for head and neck cancers (132); is effective in preventing squamous cell carcinoma of the cervix and skin when administered with IFN-α; and is being evaluated for its ability, alone and in combination with IFN-α, to prevent bronchial neoplasia (133, 134). Results regarding the prevention of colon cancer by retinoids, however, are mixed. The most encouraging studies have reported a reduction in the frequency of colon tumors (135) and a delay in tumor development in rats dosed simultaneously with DMH and 13-cis-retinoic acid (136); a reduction in the number of tumors per rat in rats dosed with DMH and retinyl palmitate (137), and reduction of aberrant crypt foci in AOM-induced rat colon (85, 137). However, several studies reported no beneficial effects on colon cancer in rats from administration of retinoids (134).

Although it is well established that the retinoids generally regulate cell differentiation in epithelial tissues, the exact mechanisms by which these compounds inhibit neoplastic transformation are not clear (4). Retinoids may inhibit cell transformation and tumor growth in part via their effects on TGF-β, a polypeptide that suppresses proliferation and induces differentiation of both normal and malignant cells. Retinoids stimulate the synthesis of isomers of TGF-β and induce the formation of TGF-β receptors in healthy and malignant cells (138). The discovery of specific nuclear RARs, present in many cells and tissues, suggests that the retinoids act by interfering with genes that regulate differentiation. The RARs belong to the large family of receptors that includes steroid, thyroid hormone, and vitamin D receptors; these receptors are proteins that function as trans-acting transcription modulating factors (139). Elucidation of the genes controlled by the RARs, combined with the synthesis of new retinoids that activate specific RARs, might enable researchers to target the effects of retinoids to certain tissue or tumors (139), including colorectal tumors.

In addition to their differentiation effects, the retinoids have been reported to suppress metastasis by decreasing the ability of neoplastic cells to invade across the basement membrane and by inhibiting enzymes involved in degradation of the basement membrane and the extracellular matrix. Further, retinoids appear to inhibit angiogenesis in model systems (140). Confounding the elucidation of the mechanism of retinoid activity are observations of retinoid enhancement rather than inhibition of carcinogenesis. Verma et al. (141) found that vitamin A increased mouse skin tumors induced by 7,12-dimethylbenz(a)anthracene alone, although it decreased those tumors initiated by 7,12-dimethylbenz(a)anthracene and promoted by TPA.

Delta-noids. Delta-noids have attracted increasing interest as potential chemopreventive agents. In a recent study, for example, the vitamin D analogue 1α,25-dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol (Ro 24-5531) reduced both the incidence and number of N-nitroso-N-methylurea-induced mammary tumors in rats, enhanced the ability of tamoxifen to reduce total tumor burden, and was a potent inhibitor of human breast cancer cell proliferation in vitro (131). Evidence also supports the suggestion that delta-noids may be a protective factor in colorectal cancer development. 1α,25-Dihydroxyvitamin D3, the biologically active metabolite of vitamin D3, is an important modulator of the absorption and metabolism of calcium and may influence cell proliferation and differentiation because of its influence on calcium metabolism. It has been shown to inhibit proliferation and to enhance differentiation in colon cell lines, both in vitro and in vivo (142, 143). Because the hypercalcemic effect of vitamin D3 precludes clinical use of the compound as a chemopreventive agent, analogues of vitamin D3 with lower toxicities have been developed, and their biological activities have been investigated and compared with the parent compound. For example, two synthetic analogues, 1,25S,26-trihydroxy-Δ22-vitamin D3 and 1,25S-dihydroxy-Δ16-23-yne-vitamin D3, have been reported to be five and ten times, respectively, more efficient than vitamin D3 in suppressing growth of human colon adenocarcinoma-derived Caco-2 cells (144). Although 1,25S-dihydroxy-Δ16-23-yne-vitamin D3 also induced differentiation in Caco-2 cells effectively, use of this analogue carries a higher risk of hypercalcemia than does the use of 1,25S,26-trihydroxy-Δ22-vitamin D3 (144). Several vitamin D analogues have been investigated by the NCI as potential candidates for chemoprevention testing in animal models for colorectal cancer, particularly in combination with calcium.

Antioxidants. In addition to their antimutagenic effects, the antioxidants noted earlier have the potential to inhibit tumor promotion by virtue of their ability to inactivate free radicals and reactive oxygen species. Substantial experimental evidence indicates that free radicals, particularly those derived from molecular oxygen, play a role in tumor promotion (15, 145). Free radical-generating dialkyl peroxides and hydroperoxides such as benzoyl peroxide, decanoyl peroxide, and cumene hydroperoxide are very active promoters of skin tumors in mice (146, 147). Further, phorbol diesters such as TPA, which are strong epidermal tumor promoters, act as stimulators of reactive oxygen metabolism, as monitored by oxygen radical production (145). Defense mechanisms present in cells for coping with free radicals include antioxidants such as GSH and vitamins C and E, as well as enzymes such as superoxide dismutase, catalase, and peroxidases (145). Tumor promoters such as TPA have been observed to bring about a rapid, sustained decrease in activity of the enzymes that contribute to cellular antioxidant defenses (15, 145). Antioxidants have been reported to block the generation of oxygen radicals by phorbol diesters (148), and phenolic antioxidants such as quercetin and vitamin E can inhibit tumor promoters both in vitro and in vivo (145).
Food Constituents in Relation to the Colorectal Tumorigenesis Model

Epidemiological studies suggest that diet is an important factor in the development of colorectal cancer, particularly certain types of foods, including high-fat foods and cooked meat (which may enhance carcinogenesis) and high-fiber foods such as vegetables, fruits, cereals and grains (which tend to lower cancer risk). Laboratory and animal studies support these epidemiological observations (149–154). The development of a preliminary genetic model for colorectal tumorigenesis provides the opportunity to consider and possibly elucidate links between such epidemiological dietary risk factors and the molecular biology of colorectal cancer. For example, the Weinstein hypothesis (155), described below, speculates on how dietary fat intake might influence interactions at the molecular level in the intracellular cascade that leads to cell replication (152). Although exploration of such links is just beginning, increased knowledge of the molecular mechanisms by which food constituents affect colorectal cancer risk will potentially increase our ability to inhibit those mechanisms by the use of appropriate chemopreventive agents.

Dietary Fat

Experimental studies provide evidence that high-fat diets increase colon cancer by acting on tumor promotion (151, 153, 154). Also, evidence exists that certain intestinal flora enhance colon cancer (155). In a novel hypothesis to explain the roles of dietary lipids and intestinal microflora in the etiology of human colon cancer, Weinstein and colleagues (155) have proposed that fat is important in colon carcinogenesis because it is a source of DAG, produced by the action of intestinal microflora on dietary lipids. Intracellular DAG, an important part of the cascade that leads from ras activation or from growth factors to protein kinase C activation, protein phosphorylation, and cell turnover (152), could enter the colonic epithelium and thus stimulate the proliferation of colonic epithelial cells by activating protein kinase C, a calcium-dependent enzyme that plays a key role in growth control (101, 152, 155, 156). This hypothesis is supported by results from an in vitro study demonstrating that bacteria in human fecal specimens can produce DAG, that this activity is enhanced by levels of specific bile acids (which are dependent on dietary lipids), and that this activity varies considerably among individuals; a 27-fold interindividual variation in DAG production was observed (155). Further, results of a recent study indicate that both the amount and type of DAGs produced may depend on the nature of dietary fiber as well as dietary lipids. Dietary wheat bran, but not corn or oat bran, significantly decreased the levels of fecal DAGs in premenopausal women (157), an interesting result in view of the fact that wheat bran appears to inhibit colon tumor development in animal models more consistently than other fiber sources (158). From a point in the colorectal tumorigenesis model. Therefore, chemopreventive agents that may be beneficial in terms of meats, fowl, and fish during cooking (particularly at high temperatures) form DNA adducts and are potent mutagens and carcinogens (160–164). Quantitative risk assessments have estimated that consumption of HAAs may result in 1 cancer case/1000 individuals, based on the estimated mean lifetime exposure to HAAs for the United States population and on animal toxicity data (160). At least 17 different HAAs have been identified in cooked muscle meats (160, 165). Of greatest concern is the class of HAAs known as aminoimidazo-azaarenes; four of these, IQ, MeIQ, 8-MeIQx, and PhIP, cause a variety of tumors when fed to rodents at levels from 0.03–0.06% of the diet. The most abundant HAA in cooked meats, PhIP, causes lymphomas in mice and mammary and large intestine tumors in rats (160, 162, 166, 167). Studies with nonhuman primates indicate that IQ, 8-MeIQx, and PhIP are rapidly absorbed after ingestion; most of the material absorbed is metabolized into active carcinogens, and only small amounts are excreted unchanged. DNA adducts of IQ and PhIP have been detected in the WBC of monkeys fed these HAAs (160).

The metabolism of HAAs by humans appears to be similar to that of nonhuman primates. One study demonstrated that only 2–5% of the MeIQxs in cooked ground meat consumed by human volunteers was excreted unchanged in the urine, indicating that MeIQx is efficiently absorbed and bioavailable (168). Mutagenic activity has been demonstrated in both the feces and urine of individuals who consume fried or cooked meats (168–172). During metabolism, HAAs undergo hepatic N-oxidation, followed by N-glucuronidation. The resulting metabolites can be transported to the colon lumen, deconjugated by bacterial ß-glucuronidas, and reabsorbed into the mucosa, where they are substrates for O-acetylation, producing N-acetoxyarylamines that are readily able to form DNA adducts (163, 173, 174). It appears that the metabolism of HAAs may be under genetic control (152). A recent case-control study investigated the distribution of acetyltransferase and cytochrome P4501A2, two genetically polymorphic enzymes related to HAA metabolism. Acetyltransferase is involved in O-acetylation, and high activity of this enzyme has been associated with increased susceptibility to colorectal cancer; cytochrome P4501A2 is the principal liver enzyme involved in HAA N-oxidation (163). Study results indicated that both acetylator status and N-oxidation status predict risk; individuals who are both fast acetylators and fast N-oxidizers have nearly three times the risk of developing colorectal cancer as individuals who are both slow acetylators and slow N-oxidizers. Forty % of colorectal cancer patients and 33% of polyp patients, but only 16% of controls, possessed the rapid-rapid phenotype, suggesting that these metabolic polymorphisms can be used to predict individual susceptibility to colorectal carcinogenesis (163, 174).

Although it has been clearly established that HAA-DNA adducts are formed in vivo and, therefore, may have the potential to increase cancer risk (163), much remains to be learned about which specific adducts may be linked to colorectal tumorigenesis and what roles they may play. For example, only approximately 40% of human colorectal cancers are associated with K-ras activation (175). HAA-induced colon cancers in rodents do not frequently show ras mutations (164). Thus, a mechanism involving other oncogenes in rat colon cancers induced by HAAs may correspond to a mechanism(s) for those cases of human colon cancer in which K-ras is not detected (164). HAA-DNA adducts could potentially influence the development of mutations at any point in the colorectal tumorigenesis model. Therefore, chemopreventive agents that may be beneficial in terms of
reducing colorectal cancer risk associated with HAAs in cooked foods include compounds such as S-allyl-L-cysteine, NAC, oligopiraz, d-limonene, and DHEA that block or suppress mutation, often by enhancing carcinogen detoxifying enzymes, as well as antioxidants (e.g., vitamin E, curcumin, and polyphenols such as ellagic acid; Ref. 36). However, investigations of specific actions of these chemopreventive agents with respect to HAAs per se remain to be carried out.

Conclusion

Although tremendous progress has been made in the field of chemoprevention since its beginning in the early 1980s, much remains to be learned about the interactions of promising chemopreventive agents with the molecular and cellular processes that contribute to carcinogenesis, both generally and for specific types of cancer. The proposed model for colorectal tumorigenesis, although it may be incomplete, has provided a framework for designing innovative, potentially effective chemopreventive approaches for colorectal cancer. Because many of the chemopreventive agents that show promise for colorectal cancer may produce their effects by more than one mechanism of action, determining the best chemopreventive approaches is challenging. The use of chemopreventive substances or mixtures of substances from natural sources (e.g., phenolic compounds in green and black tea, d-limonene in citrus oils, and ellagic acid in fruits and vegetables) is a desirable approach that appears to be particularly appropriate for colorectal tumorigenesis. However, the development of clear guidelines by the Food and Drug Administration for the preparation of such substances and for their testing in clinical trials will be important for fostering chemoprevention research using such agents. Further, evaluation of the effectiveness of potential chemopreventive agents, whether naturally occurring or pharmaceuticals, can be facilitated by the development of standardized SEBs that are valid and reliable and that can be used in short-term intervention trials. The characteristic morphological changes in colorectal cancer, as well as the proliferative and biochemical parameters that correlate closely with the progression of intraepithelial neoplasia, provide excellent candidates for SEBs for this disease. The development of SEBs is receiving significant research emphasis not only for colorectal cancer but also for cancers at other sites.

As research continues to clarify the mechanisms of action of chemopreventive agents and to unravel the steps in carcinogenesis at various cancer sites, a sharper picture will emerge of the best options available for cancer chemoprevention in specific clinical circumstances. While our understanding of the fit between chemopreventive mechanisms and carcinogenesis is evolving, development of chemopreventive regimens based on empirical data from epidemiological and experimental studies will continue to serve as the best practical approach to design potentially effective chemoprevention trials.

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