MINIREVIEW

Commensal Bacteria, Redox Stress, and Colorectal Cancer: Mechanisms and Models

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The potential role for commensal bacteria in colorectal carcinogenesis is explored in this review. Most colorectal cancers (CRCs) occur sporadically and arise from the gradual accumulation of mutations in genes regulating cell growth and DNA repair. Genetic mutations followed by clonal selection result in the transformation of normal cells into malignant derivatives. Numerous toxicological effects of colonic bacteria have been reported. However, those recognized as damaging epithelial cell DNA are most easily reconciled with the currently understood genetic basis for sporadic CRC. Thus, we focus on mechanisms by which particular commensal bacteria may convert dietary procarcinogens into DNA damaging agents (e.g., ethanol and heterocyclic amines) or directly generate carcinogens (e.g., fecapentaenes). Although these and other metabolic activities have yet to be linked directly to sporadic CRC, several lines of investigation are reviewed to highlight difficulties and progress in the area. Particular focus is given to commensal bacteria that alter the epithelial redox environment, such as production of oxygen radicals by *Enterococcus faecalis* or production of hydrogen sulfide by sulfate-reducing bacteria (SRB). Superoxide-producing *E. faecalis* has conclusively been shown to cause colonic epithelial cell DNA damage. Though SRB-derived hydrogen sulfide (H_2S) has not been reported thus far to induce DNA damage or function as a carcinogen, recent data demonstrate that this reductant activates molecular pathways implicated in CRC. These observations combined with evidence that SRB carriage may be genetically encoded evoke a working model that incorporates multifactorial gene-environment interactions that appear to underlie the development of sporadic CRC. Exp Biol Med 229:586–597 2004

Key words: colorectal cancer; chromosomal instability; colonic microbiota; redox stress; *Enterococcus faecalis*; sulfate-reducing bacteria

Introduction

Each year on a worldwide basis, approximately 940,000 persons are diagnosed with colorectal cancer (CRC), and of these more than 500,000 die from its complications (1). Although rare in developing countries, CRC is the second most frequent malignancy in affluent nations. Greater than 80% of CRCs occur sporadically, and these have convincingly been shown to arise from adenomatous polyps through the gradual accumulation of mutations in genes such as *APC*, K-*ras*, *TP53*, *CTNNB1*, *MADH4/SMAD4*, *TGFBR2*, and mismatch repair (2–5). Genetic mutations followed by clonal selection under environmental constraints result in the transformation of normal cells into malignant derivatives (2, 6). The mechanism(s) by which mutations occur for sporadic CRC remains a central question in the field of carcinogenesis.

The first attempts to associate commensal bacteria with CRC relied on cultures of fecal bacteria from people with differing risks for CRC (7–9). The goal was to characterize specific organisms that conferred an altered risk for CRC. This seemed reasonable because intestinal cancer occurs

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1535-3702/04/2295-0001$15.00

almost exclusively in the colon where metabolically active bacteria are in direct proximity to mucosal surfaces at densities of 10^{11} colony-forming units per gram of fecal material. Unfortunately, the studies proved difficult and provided, at best, equivocal results (7, 9, 10). Conceptual problems arose as the enormous complexity of the fecal microbiota was recognized with hundreds of species, many of which could not be recovered by cultivation (11, 12). In addition, distinct luminal and mucosal-associated habitats were recognized (13, 14) and previously unappreciated host-specific effects on the fecal microbiota were identified using molecular techniques (15, 16).

In this review, potential roles for commensal bacteria in colorectal carcinogenesis will be explored. Although this topic has previously been considered (17–19), it has as yet to be placed in the context of genetic, enzymatic, and environmental factors associated with CRC. Genetically engineered animal models of CRC that implicate the colonic microbiota will be described along with mechanisms by which commensal bacteria might generate carcinogens, convert dietary procarcinogens into DNA damaging agents, or evoke endogenous redox stress. As tumor formation and progression may be independently regulated, sulfate-reducing bacteria (SRB) will also be considered as a chronic proliferative stimulus. Although SRB are but one of several commensal bacteria that could contribute to proliferative, antiapoptotic, or toxic epithelial effects (19–27), they are featured because their ability to modulate intestinal redox status and their potential role in CRC have not been previously reviewed.

Finally, although numerous toxicological effects of colonic commensal bacteria are known, we will focus on those capable of leading to epithelial cell DNA damage. This approach was chosen because bacterially induced mutagenesis easily reconciles with our current understanding of the genetic basis for sporadic CRC. If genetic mutation is considered essential to the initiation of sporadic colonic neoplasia (4, 5, 28), then the role for commensal bacteria will likely remain unclear until bacterially mediated mechanisms for DNA damage (or protection) are defined. For example, commensal bacteria can metabolize fecal steroids and generate short-chain fatty acids, but these activities are not known to damage eukaryotic cell DNA (19, 22–27). Similarly, intestinal pathogens that are not commensals may have proliferative, antiapoptotic, or toxic epithelial effects (20, 21, 29) but are not considered promutagenic. Other pathogens, including strains of *Escherichia coli* that produce heat-stable enterotoxins, may exert antiproliferative effects that lower the risk for CRC (30), but mechanisms by which DNA might be protected from damage are not clear. Furthermore, viruses were not considered in order to focus on commensal bacteria, although the human polyomavirus JC virus has been associated with CRC and promotes chromosomal instability (CIN) *in vitro* (31, 32). The following discussion is limited to intestinal commensals that may promote mutagenesis or act in concert with promutagenic bacteria to drive the cellular evolution that leads to a malignant phenotype.

Colorectal Carcinogenesis

Essential features of neoplastic cells include self-sufficiency in growth signals, insensitivity to growth inhibition, evasion of apoptosis, limitless replicative capacity, angiogenesis, and tissue invasion (6, 33). Fundamental to the acquisition of these traits is genomic instability, a process that leads to cellular evolution and can result in CRC (28). Chromosomal instability is the most common form of somatic genomic instability and is typified by rearrangements, losses and gains of large DNA fragments, aneuploidy, and loss of heterozygosity (3, 28, 34–36). This form of instability is found in >80% of sporadic CRC (3). The mechanism by which CIN develops remains unknown.

In contrast to most sporadic disease, inheritable forms of CRC, such as hereditary nonpolyposis colorectal cancer, typically demonstrate microsatellite instability (MIN; Ref. 37). This form of genomic instability is distinct from CIN, defined by numerous mutations in repetitive DNA sequences, and results from defects in DNA mismatch repair. Colorectal tumors express CIN or MIN, but rarely both. Transforming growth factor βs (TGF-βs) are potent inhibitors of normal cell growth, and mutations in *TGFBR2* are found in 90% of MIN tumors (38). Conversely, Smad2, Smad3, and Smad4 are intracellular proteins that transduce TGF-β signals and, at least for *Smad4*, appear more often mutated in microsatellite stable forms of sporadic CRC (39, 40).

Another unresolved issue concerning CRC involves the permissive role of type 2 cyclooxygenase (COX-2), an inducible enzyme whose expression is associated with a poor prognosis in CRC (41). Cyclooxygenase-2 catalyzes sequential reactions leading to the dioxygenation of polyunsaturated fatty acids (42). Prostaglandin (PG) H_2 is the COX-2 product of arachidonic acid and a precursor for the family of prostaglandins that includes PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane. In CRC and precursor adenomas, COX-2 is most often localized to submucosal dendritic cells or macrophages and not the epithelium (43, 44). The importance of COX-2 in CRC is evident from inhibitor studies that show effective chemoprevention (42). The mechanism for this effect, however, remains obscure. Recently, COX-independent effects were proposed as an explanation (45), but deletion and upregulation of *Cox* genes in animal models of intestinal neoplasia suggest these enzymes, independent of any effects caused by COX-inhibiting drugs, are directly important to colorectal carcinogenesis (46, 47). The ability of commensal bacteria to alter COX-2 expression remains largely unexplored.

Finally, environmental factors such as physical activity, diet, ethanol consumption, and bacterial catabolites or toxins are believed to play a significant role in CRC (48, 49).

Colorectal cancer incidence varies more than 10-fold across the globe with rates increasing rapidly in groups that migrate from low- to high-incidence areas (50). It has been estimated that environmental factors, including diet, account for up to 90% of this variation (51). Although the most consistent dietary influence on CRC risk appears to be simple caloric restriction (49, 52), and possibly red meat intake (53), relationships among other environmental factors, genomic instability, COX-2 expression, and colonic bacteria remain to be determined.

Animal Models Implicating Commensal Bacteria in CRC

Gene inactivation studies have provided substantial insight into complex pathological processes like cancer. The first genetically engineered murine model for CRC was discovered by random mutation using an alkylating agent (54). Affected mice developed intestinal adenomas and were referred to by the acronym "Min" for multiple intestinal neoplasia. Genetic analyses identified a mutation in the adenomatous polyposis coli gene (*APC*) whose product modulates oncogenic *Wnt* signal transduction through β-catenin (55). Additional murine models with mutations in WNT signaling have been described, each of which also leads to intestinal adenomas (56). The Min model is analogous to familial adenomatous polyposis coli, a hereditary form of human CRC due to the germline inactivation of *APC*. The adenomatous polyposis coli gene is also often inactivated in sporadic CRC and considered a frequent early event in the progression of adenomas to cancer (55). The primary location of most adenomas in the Min model, however, is in the small intestine, unlike sporadic human CRC where tumors occur in the large intestine.

Other genetically engineered models of intestinal neoplasia include knockouts in mismatch repair genes, *Smad3*, *Il-10*, $G_{\alpha i2}$, *Muc2*, $T_{cr\alpha}$, *Cdx2* (56–61); double knockouts in *Smad4* with *APC*, *Tgfβ-1* with *Rag2*, *Il-2* with *β2m*, *Gpx1* with *Gpx2*, and *TCRβ* with *p53* (62–65), and expression of cloned bone morphogenetic protein-4 inhibitor noggin (66). Of note, many, but not all, of these models exhibit inflammatory bowel disease. For a few models, the influence of commensal bacteria on inflammation and tumor formation has been investigated. Under germ-free conditions, intestinal inflammation was significantly decreased and tumors did not form for *Il-10* knockout mice or double knockouts of *Tgfβ-1* with *Rag2*, *TCRβ* with *p53*, or *Gpx1* with *Gpx2* (63, 67–69). Although Min mice have little intestinal inflammation, germ-free animals showed a 50% reduction in the number of small intestinal adenomas suggesting commensal bacteria also potentiate tumor formation in this model (70).

These studies suggest that commensal colonic microbiota are important to the induction of inflammation and development of CRC, although not all bacteria appear equally capable of causing (or protecting against) pathology (69, 71). For example, *Il-10* knockout mice monoassociated with *Enterococcus faecalis*, a human intestinal commensal, develop colitis and tumors, whereas numerous other commensal and pathogenic bacteria and yeast fail to produce any intestinal pathology (69). In contrast, *Lactobacillus* spp. appear to protect against inflammation and cancer in this same model (72, 73). In aggregate, these findings suggest a significant role for commensal bacteria in intestinal inflammation and tumor formation.

These genetically engineered models were all developed in mice, and the differences between rodent and human commensal microbiota have as yet to be well characterized. Several significant murine pathogens are not known to colonize humans. For example, *Helicobacter hepaticus* and *Citrobacter rodentium* are both associated with enterocolitis, intestinal hyperplasia, and tumor formation in mice (74), and have been linked to CRC in several animal models. *H. hepaticus*, which is known to cause necrotizing hepatitis that progresses to hepatocellular carcinoma, colonizes the murine intestine. In immunocompetent mice, this leads to mild intestinal inflammation and epithelial hyperplasia (75). *Rag2* knockout mice colonized with *H. hepaticus* rapidly develop colitis and colon cancer (76), an effect largely ameliorated by IL-10 producing lymphocytes (77). Bacterial virulence traits responsible for colitis, however, remain to be defined. Another murine pathogen that causes proliferative colitis is *C. rodentium* (78). Min mice infected with *C. rodentium* at 1 month of age showed a 4-fold increase in the number of colonic adenomas after 6 months compared to uninfected Min mice (29). Colonic adenomas in infected mice were largely restricted to the distal colon where *C. rodentium*–induced hyperplasia occurred. The mechanism for epithelial cell hyperproliferation or carcinogenesis is not fully understood. B-cell–mediated immune responses appear important for control of *C. rodentium* infection, and the type IV pilus facilitates colonization (79, 80).

Unfortunately, no single animal model mimics human sporadic CRC or associated CIN (56). Many models, however, still need chromosomal analysis of tumors and evaluation under gnotobiotic conditions. The association of genetically engineered mice with defined commensals should permit examination of mechanisms by which commensal bacteria may provoke CIN, induce COX-2, or affect dietary factors implicated in colorectal carcinogenesis. For example, the effect of redox stress by commensal bacteria on COX-2 expression and induction of CIN could be evaluated using gnotobiotic *Gpx1-Gpx2* or *Muc2* knockout mice. Bacterial antigen stimulation leading to inflammation, COX-2 expression, or CIN might be addressed using *IL-10*, *IL-2-β2m*, $T_{cr\beta}$, or $G_{\alpha i2}$ knockout models. Finally, investigation into the role of the intestinal microbiota on modulating Tgf-β signaling could be approached using *Tgfβ1-Rag2* and *Smad3* or *Smad4*

Table 1. Crucial Research Needs to Better Understand the Role of Commensal Bacteria in the Initiation or Progression of Colorectal Cancer

Development of murine models that better mimic the multigenetic origin of sporadic CRC in humans.
Study of current animal models to identify the components of the commensal microbiota that promote intestinal inflammation and CRC; this should be followed by characterizing relevant microbial traits.
Exploration of mechanisms by which commensal bacteria or their products might induce or otherwise alter COX-2 expression.
Exploration of mechanisms by which commensal bacteria or their products might act as mutagens.
Characterization of the effect of genetic background on the composition of the commensal microbiota.

knockouts. These ideas represent only one of several potential areas for research focus (Table 1).

Activation of Procarcinogens by Commensal Bacteria

The colonic microbiota is composed of hundreds of microbial species that form a metabolically complex ecosystem. This milieu benefits the host by excluding exogenous pathogens, providing nutrients as by-products of metabolism, and inactivating toxins. Alternatively, the colonic microbiota may be detrimental by promoting inflammation or converting innocuous compounds into metabolites permissive to inflammation or tumorigenesis. It should be emphasized that specific food items or nutrients that might cause CRC have not been identified. Much information, however, is available concerning enzymatic activities that are expressed by colonic bacteria and might generate carcinogens. These activities include β-glycosidases, β-glucuronidases, azo- and nitroreductases, arylsulfatases, and alcohol dehydrogenases (18, 81). Despite many efforts, investigators have as yet to directly link these metabolic activities to sporadic CRC. Nor is there clear evidence to show how these activities might explain the epidemiology of CRC. Despite this, several lines of investigation are reviewed to highlight difficulties and progress in this area.

Ethanol, Acetaldehyde, and Folate. Multiple epidemiological studies implicate two dietary factors, ethanol and folate, in an altered risk for CRC (49, 82–85). The postulated mechanisms for folate deficiency increasing CRC risk are (i) altered gene promoter methylation (86, 87), (ii) increased single- and double-stranded DNA breaks (88), and (iii) misincorporation of uracil for thymine during DNA synthesis leading to mutations (89, 90). Ethanol directly interferes with folate availability and independently produces high concentrations of acetaldehyde, a known chemical carcinogen (91), in the colon *via* bacterial metabolism. Acetaldehyde is thought to promote mutagenesis by inactivating cellular proteins important to DNA repair such as O^6-methylguanine transferase (92, 93), inhibiting methyltetrahydrofolate or methionine synthase to trap folate as 5-methyltetrahydrofolate, or by direct cleavage to reduce intestinal absorption of folate (94). Diets high in ethanol and low in folate (and methionine) are considered "methyl-poor" and confer a markedly greater risk for adenomas and CRC than "methyl-rich" diets (82, 95). Of note, the additive effects of ethanol and folate are negated by aspirin, an irreversible inhibitor of COX isoforms (95).

In addition to these issues, it is possible that folate status may not be entirely determined by dietary intake. Colonic bacteria can synthesize several vitamins *de novo* including folate. Some portion of bacterially derived folate can be absorbed (96). Estimates suggest <7% of tissue folate is derived from bacterial synthesis (97). Whether such a proportion is sufficient to protect against DNA damage after dietary restriction remains to be determined. Efforts to modulate bacterial folate synthesis through dietary fiber to augment colonic fermentation or by using sulfa derivatives to inhibit bacterial synthesis have yet to define fully the interplay between diet, fecal bacteria, host genotype, and folate (84, 98–100).

Many colonic bacteria express alcohol dehydrogenase (ADH). This enzyme contributes to the fermentation of sugars into ethanol. However, if excess ethanol is present, as occurs after moderate alcohol consumption, microbial ADH activity can be reversed and lead to the production of acetaldehyde. This phenomenon has been observed in rats and piglets fed ethanol where increased concentrations of acetaldehyde are found in colonic contents (94, 101). These studies suggest sporadic CRC may occur, in part, at the convergence of environmental, genetic, and metabolic variables with the latter dictated by commensal bacteria. However, evidence to link folate depletion and ADH metabolism to epithelial cell mutations, genetic instability, or CRC is lacking.

Heterocyclic Amines. Fish and beef generate promutagenic heterocyclic amines (HCAs) during cooking (102). These molecules are carcinogenic in mice, rats, and monkeys producing hepatic, intestinal, and mammary tumors (103, 104). The aminoimidazoazaarenes are a major group of heterocyclic amines in the human diet (102). As with other heterocyclic amines, these compounds are only genotoxic after activation to electrophilic derivatives that form DNA adducts (105). A variety of host drug-metabolizing enzymes can activate (and detoxify) heterocyclic amines including CYP1A2, *N*-acetyltransferase, sulfotransferase, prolyl tRNA synthetase, phosphorylase, and COX isomers (105, 106). In a recent case-control analysis, associations were not found between CRC risk and polymorphisms in these genes (107). This comprehensive study, however, failed to consider commensal bacteria and their potential impact on heterocyclic amine activation, an effect independent of host genotype.

One HCA, 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline (IQ), is produced through the pyrolysis of creatinine

with sugars. IQ is s a procarcinogen and becomes mutagenic in the presence of hepatic microsomes to generate 200–400 revertants per nanogram in the *Salmonella typhimurium* TA98 assay (108). Anaerobic colonic bacteria can convert IQ to 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline-7-one (HOIQ), a direct-acting mutagen (109, 110). *Eubacterium* spp specifically metabolize IQ to HOIQ along with undefined commensal bacteria from mice, rats, and humans (111, 112). These commensal bacteria can strongly influence IQ-induced DNA damage for colonic cells (and hepatocytes) as measured by the alkaline single-cell gel electrophoresis assay (113). DNA from axenic rats exhibited significantly fewer alkaline-labile breaks than rats colonized with conventional murine or human commensal bacteria. In contrast, other intestinal commensals, including *Bifidobacterium longum* and lactobacilli, appear antagonistic to the mutagenic effects of IQ (114, 115). Mechanisms underlying these observations are unclear but may involve inactivation of IQ or direct binding of IQ to bacteria. Judgments about the significance of IQ or HOIQ in promoting CRC, however, still await appropriately designed clinical studies.

Direct Production of Mutagens by Commensal Bacteria

Fecapentaenes. The fecapentaenes are a family of ether-linked polyunsaturated lipids with potent *in vitro* mutagenic effects (116). Fecapentaenes are produced by *Bacteroides* spp at detectable concentrations in the colon. The mechanisms for genotoxicity are unknown, but some evidence indicates oxidative damage to DNA can occur through radical mechanisms. Peroxidation by COX isoforms can also generate hydroxyl radical when iron is available as a catalyst (117, 118). Target cells with high concentrations of 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG), a marker for oxidatively damaged DNA, support this hypothesis (119). Alternatively, fecapentaenes are reactive electrophiles that may alkylate DNA to form mutagenic adducts (116). *In vitro* and *in vivo* studies using the 12-carbon fecapentaene showed DNA damage in colonic epithelial cells and tumor promotion (120, 121). These and other suggestive data resulted in carefully designed case-control studies to test the hypothesis that increased excretion of mutagenic fecapentaenes might be a cause for sporadic CRC (122) or adenomatous polyps (123). Surprisingly, no association was found between fecal fecapentaenes and colonic tumors. Although investigators recognized other non-fecapentaene fecal mutagens may have confounded analyses (124), an explanation for these negative results was not apparent. Since this work, further study on fecapentaenes has been minimal. These results emphasize the need for well designed clinical trials to confirm or refute suggestive *in vitro* and animal data on fecal mutagens.

Oxygen Radicals. Oxidative damage produced by endogenous redox sources is a potentially important mechanism for somatic mutations that give rise to cancer (125). Endogenous genomic stress originates from reactive oxygen intermediates that directly attack DNA or generate reactive intermediates. In biological systems, the most common reactive oxygen species are superoxide, hydrogen peroxide, hydroxyl radical, and peroxynitrite. Superoxide is a transient anionic radical generated by the univalent reduction of oxygen and, quite importantly, participates in the formation of other reactive oxygen species. Hydrogen peroxide is a two-electron reductant of oxygen and, therefore, not a true radical. Although hydrogen peroxide has a long half-life, in the presence of superoxide, iron, or copper it can readily generate hydroxyl radical (126). This three-electron reductant of oxygen is extremely reactive and usually damages the first molecule it encounters. Finally, peroxynitrite is produced when superoxide reacts with nitric oxide. This potent oxidant can decompose into other radicals and cause DNA strand breakage or oxidize and nitrate bases (127).

Although several reactive oxygen species can damage DNA, hydrogen peroxide is the only one that is stable enough to diffuse into cells where, in the presence of transition metals, hydroxyl radical can be generated (128). The abundant production of 8-oxo-dG in cells treated with hydrogen peroxide is an indicator of this facile process. Other biological targets besides DNA obviously exist for reactive oxygen species, most notably polyunsaturated fatty acids in eukaryotic phospholipid membranes. Bis-allelic hydrogens in these molecules are susceptible to radical abstraction, a process that can result in chain reactions and produce enormous numbers of oxidized fatty acids (129). Breakdown products include diffusible electrophilic aldehydes such as malondialdehyde, 4-hydroxy-2-nonenal, and 4-oxo-2-nonenal, all of which generate mutagenic etheno-DNA adducts (130, 131).

One potential mechanism for CIN involves oxygen radical generation by commensal bacteria leading to ongoing epithelial cell DNA damage. This hypothesis was formulated following *ex vivo* observations of abundant hydroxyl radical production by normal stool (132). Others subsequently confirmed these initial findings (133–135). This concept is also consistent with genomic instability arising from dietary procarcinogens activated by colonic radicals (136, 137).

Several years ago, *Enterococcus faecalis* was found to produce extracellular superoxide (138). This oxidative phenotype depended on membrane-associated demethylmenaquinone and was the result of dysfunctional microbial respiration. Exogenous fumarate or hematin suppressed superoxide production by providing substrate for fumarate reductase or reconstituting cytochrome *bd* (139). *Ex vivo* analysis of colonic contents from rats colonized with *E. faecalis* revealed hydroxyl and sulfur-centered (or thiyl) radicals using electron spin resonance (ESR) spin trapping (139, 140). The *in vivo* production of hydroxyl radical by *E. faecalis*, which arises from superoxide, was confirmed by measuring the aromatic hydroxylation of phenylalanine and

phenyl *N*-tertbutylnitrone in colonized rats (141). These compounds are targets for hydroxyl radical and form specific hydroxylated products that are easily detected. Rats colonized by superoxide-producing *E. faecalis* generate 15- to 25-fold greater concentrations of hydroxylated aromatic targets in urine than control rats colonized with an isogenic strain showing attenuated superoxide production (120).

These findings suggested endogenous reactive oxygen species formed by *E. faecalis* near the oxygenated luminal surface of colonocytes could be a source of CIN. In the mildly acidic environment of the colon, superoxide would spontaneously disproportionate to hydrogen peroxide and accumulate to micromolar concentrations (141, 142). Upon passive diffusion into epithelial cells, hydrogen peroxide can form hydroxyl radical near DNA through iron-catalyzed reactions and cause DNA-protein cross-linking, DNA breaks, and base modifications (128, 143). In a short-term model of intestinal colonization, the comet assay was used to demonstrate this effect on colonic epithelial cells by superoxide-producing *E. faecalis* (142). It remains to be determined whether commensal enterococci also oxidize cellular fatty acids to form secondary electrophiles and mutagenic DNA adducts. This would be another mechanism by which endogenous redox activity by commensal bacteria might promote CIN. Although the only human study to examine intestinal colonization by superoxide-producing enterococci failed to associate these bacteria with adenomas or CRC (16), colonization was not stable over time and likely confounded the findings. Proper examination of potential associations will likely require a long-term prospective study of relevant colonic bacteria using molecular-based approaches.

Sulfate-Reducing Bacteria and Hydrogen Sulfide

Sulfidogenic bacteria are often members of the normal colonic microbiota and can have a major impact on bacterial metabolism through their disposal of the H_2 reducing equivalents generated from fermentation. Although morphologically diverse and metabolically versatile, SRB are considered a physiologically unified group because of their ability to use sulfate (SO_4^{2-}) as an oxidant (terminal electron acceptor) for the degradation of organic matter. An equivalent amount of sulfide (H_2S) is formed per mole of sulfate reduced: $2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^-$.

Eighteen genera of dissimilatory SRB are currently recognized and classified into two physiological-ecological subgroupings (144). The Group I genera, such as *Desulfovibrio*, *Desulfomonas*, *Desulfotomaculum*, and *Desulfobulbus*, use lactate, pyruvate, ethanol, or certain fatty acids as carbon and energy sources while reducing SO_4^{2-} to H_2S. The genera in Group II, including *Desulfobacter*, *Desulfococcus*, *Desulfosarcina*, and *Desulfonema*, specialize in the oxidation of fatty acids, particularly acetate while reducing SO_4^{2-} to H_2S. Phylogenetically, most SRB align closely with other gram-negative bacteria in the delta subdivision of the Proteobacteria, whereas *Desulfotomaculum*, consisting of endospore-forming rods, groups with the *Clostridium* subdivision of the gram-positive bacteria (145, 146). Relatively little is known about the diversity and ecology of colonic SRB genera for any mammalian species.

It has clearly been demonstrated in nonintestinal anaerobic environments that when sulfate is nonlimiting, SRB generally out-compete methanogens for common growth substrates (147). It appears that a competitive relationship also exists between intestinal methanogens and SRB (148–150). In a study of 87 healthy human volunteers, three fecal SRB population groupings were recognized: Group 1 consisted of 21 persons who were strong methane (CH_4) producers in which fecal SRB were completely absent (151). In Group 2 ($n = 9$), methanogenesis occurred and low numbers of SRB (ca. 10^5/g wet weight feces) were detected, although their metabolic activities were negligible. The final group consisted of 57 volunteers exhibiting high counts of fecal SRB (up to 10^{11}/g wet weight) and complete absence of methanogenesis. The numerically predominant SRB were *Desulfovibrio* spp, which accounted for 67% to 91% of total SRB counts. Species belonging to the genera *Desulfobacter* (9% to 16%), *Desulfobulbus* (5% to 8%), and *Desulfotomaculum* (2%) were present in considerably lower numbers. Christl and co-workers (150) reported that approximately 50% of healthy human adults from European and North-American populations and 90% of rural black Africans were predominantly methane excreters and likely harbored low numbers of intestinal SRB. Cumulatively, these data indicate that SRB carriage may be genetically encoded. At the least, they demonstrate the importance of more rigorously assessing this possibility.

Hydrogen Sulfide, Inflammatory Bowel Diseases, and CRC. Although limited, several clinical studies demonstrate an association between H_2S and the development of the inflammatory bowel diseases (IBDs) and CRC (152–156). For example, fecal samples from ulcerative colitis (UC) patients were shown to harbor a greater number of SRB (153). Also, H_2S generation rates and concentrations in UC feces were significantly greater than control fecal samples (150, 152, 153, 155, 156). Kanazawa and colleagues (154) demonstrated that H_2S concentrations were also significantly greater in 13 male patients who had previously undergone surgery for sigmoid colon cancer and who later developed new epithelial neoplasia of the colon, compared to 14 males of similar age with a healthy colon. However, it is not possible from the studies above to distinguish whether the increased sulfide concentrations preceded disease or reflect an alteration of the normal microbiota as a result of chronic inflammation or surgical manipulation.

Particularly intriguing is evidence that carriage of intestinal SRB appeared to segregate according to ethnic background in the Christl *et al.* (150) study, as that outcome is consistent with both IBD and sporadic CRC being more prevalent in white populations of Northern European

descent than in populations of African descent (157–160). These observations indicate that host genetic background may influence individual variation in SRB carriage rate, evoking working models that incorporate multifactorial gene-environment interactions that appear to underlie the development of both IBD and sporadic CRC (161).

Indeed, UC and colonic Crohn's disease are associated with increased risk (approximately 5-fold) for CRC (162, 163), and it has been suggested that both IBD-associated and sporadic CRC might be the consequence of bacteria-induced inflammation (161). Both types of cancer arise from precancerous dysplastic mucosa and exhibit multistep development with multiple mutations. One obvious difference is that the majority of sporadic colon cancers arise from polyps, whereas IBD-associated cancers typically arise from flat dysplastic mucosa (161). The differential timing of mutations in *APC* versus *p53* has been suggested to underlie these pathological differences (161).

Despite the clinical links between H_2S and the development of UC or CRC, few studies have examined the impact of H_2S on intestinal epithelial cell function. Roediger and colleagues reported decreased fatty acid oxidation in colonocytes exposed to H_2S (164, 165). These H_2S-induced oxidative changes closely resembled the impairment of β-oxidation observed in colonocytes of UC patients. Christl *et al.* (166) observed a significant increase in the proliferation of cells residing in the upper crypt region of a colonic biopsy incubated for 4 hrs with 1 m*M* NaHS.

Deplancke and co-workers recently determined that H_2S concentrations in the mouse large intestine range from 0.2 to 1 m*M* (167), which are similar to the 0.3 to 3.4 m*M* H_2S concentrations reported for human feces (149, 168, 169). Intriguingly, these H_2S concentrations are 6- to 60-fold greater than previously reported H_2S concentrations (~50 μ*M*), at which complete inhibition of oxidative phosphorylation occurs (170). That such sulfide concentrations are apparently tolerated by a significant proportion of the population indicates that mechanisms of sulfide detoxification must exist, though these are poorly understood. Colonic bicarbonate secretions significantly reduce exposure of the epithelium to H_2S through conversion to anionic sulfide (171), although toxic H_2S concentrations would still exist at a pH of 7.5. Epithelial sulfide detoxification via active oxidation to thiosulfate has also been demonstrated (136, 151) and may represent a functional detoxification mechanism; however, enzymic pathways have not been identified. The subsequent involvement of rhodanese (thiosulfate:cyanide sulfurtransferase; E.C. 2.8.1.1) in colonic sulfide detoxification has been demonstrated (172). Further elucidation of colonic mechanisms of sulfide detoxification will be important if polymorphic variation in these pathways were to contribute to multigenic susceptibility to IBD-associated or sporadic CRC.

Recent functional genomic and biochemical data indicate that H_2S may perturb the precarious balance between apoptosis, proliferation, and differentiation in the intestinal epithelium (173). Deoxycholic acid, a naturally occurring modified bile acid, may contribute to colonic carcinogenesis via a similar mechanism (174). To date, H_2S has not been reported to induce DNA damage or function as a carcinogen. However, the suggested involvement of extracellular activated kinase (ERK) in H_2S-mediated mitogenic signaling and the upregulation of genes involved in mitogen activated protein kinase (MAPK) signaling (173) indicate that H_2S stimulates the Ras/Raf/MEK/ERK pathway, and the best characterized response to Ras activation is the promotion of entry into the S phase (175). It is well recognized that oncogenic activation of Ras is an important early event in colorectal tumorigenesis (176), and thus H_2S may be tumor-promoting. Consistent with this idea is additional evidence of sulfide activation of several neoplasia-associated genes, as well as the gene encoding VEGF (173). This gene plays an essential role in the progression and metastasis of numerous solid malignancies, including CRC (177). In addition, preliminary data demonstrate that sulfide stimulates NO production by the rat intestinal epithelial IEC-6 cell line (MA Ramos, HR Gaskins, unpublished). The variable mutagenic and apoptotic properties of NO are reasonably well characterized (178, 179). In contrast, the potential that intestinal sulfide may contribute to the generation of sulfur-centered radicals remains unexplored as does a potential link of the latter to carcinogenesis, despite an increasing recognition that the potent reactivity of sulfur-centered radicals renders them capable of damaging DNA under selected conditions (180, 181). Preliminary data suggest sulfur-centered radicals are a primary consequence of superoxide production by *E. faecalis* colonizing the rat colon (140). Clearly, it becomes crucial to better understand the biochemical and molecular pathways activated by sulfide in colonic epithelial cells given the combined evidence that SRB carriage may be genetically encoded and that sporadic CRC may be influenced by combinatorial polymorphisms in multiple genes responsive to environmental stimuli.

Summary

Commensal bacteria have long been suspected of contributing to CRC. Specific mechanisms, however, have proven elusive due to the complexity of the colonic microbiota and the multifactorial nature of gene-environment interactions that likely engender predisposition to CRC. Here, we have focused on mechanisms by which particular commensal bacteria may disrupt intracellular redox homeostasis and damage epithelial cell DNA. Also considered were bacterial activities that generate carcinogens or convert dietary procarcinogens into DNA-damaging agents. Although not featured, emerging molecular-based studies of the colonic microbiota indicate that its particular composition is stable within, but variable among, individuals (68, 69). Thus, host genetic background may, in some

instances, contribute to CRC indirectly through its influence on the carriage of specific bacterial groups. In other words, the genetic component of gene-environment interactions contributing to sporadic CRC may represent the combined inheritance of polymorphisms in genes that influence bacterial colonization, redox homeostasis, and epithelial detoxification or defense. Although this working model imparts focus, the paucity of information on molecular mechanisms for epithelial interactions with commensal bacteria and their metabolic products presents a challenging future.

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