Bacteria, inflammation, and colon cancer

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INTRODUCTION
Our relationship with the colonic bacterial flora has long been viewed as a symbiotic one. We provide a nutrient-rich habitat, while the bacteria play important roles in the development of the mucosal immune system, the maintenance of a physiological environment, the provision of essential nutrients, and the prevention of colonization by pathogenic bacteria\(^1,2\). Recently, the concept that normal bacterial flora are essential for the development of inflammation-induced carcinoma has emerged from studies of well-known colonic bacterial floras. This hypothesis is reviewed here because the lessons learned from investigations of the colonic flora could serve as a general guide to studies of the etiology and pathogenesis of chronic inflammatory diseases and related cancers in the gastrointestinal tract. These include conditions such as reflux esophagitis and esophageal adenocarcinoma, and Helicobacter gastritis and gastric adenocarcinoma and lymphoma, as well as inflammatory bowel diseases (IBD) and colorectal cancer.

Human colorectal carcinomas can be classified by etiology as inherited (e.g., hereditary nonpolyposis colorectal cancer due to genetic instability and familial adenomatous polyposis coli due to a mutation in the adenomatous polyposis coli gene, APC), inflammatory (e.g., Crohn’s disease and ulcerative colitis), or sporadic (accounting for greater than 80% of colorectal cancers, but poorly defined etiologically). The availability of genetically-engineered rodent models has greatly facilitated the etiologic study of colorectal cancers.

The normal bacterial flora is a prerequisite for the development of inflammation-related colorectal tumors
The role of bacteria in the development of colorectal tumors is best exemplified by the experimental dysplasia and cancer developed in the TCRβ/p53 double knockout mouse colitis model that mimics the development of adenocarcinoma in ulcerative colitis\(^3\). In conventional mice at four months of age, adenocarcinomas of the ileocecum developed in 70% of animals. However, there was no development of colonic adenocarcinoma in mice raised under germ-free housing conditions. A similar observation was also made in IL-10 knockout\(^4\) and Gpx1/Gpx2\(^5\) double knockout mice (Table 1). There is a growing list of genetically-engineered mouse models for gastrointestinal cancers, for which the effect of the normal bacterial flora on tumorigenesis has not yet been evaluated (Table 1)\(^6\).
These include knockouts of the G protein subunit alpha i2 (Gαi2)[7], Smad3[8], Muc2[9], and double knockouts of TGF-β1/Rag2[7,11] and IL-2/β2m[12].

Comparison of the results for conventional and germ-free mice indicates that hosting a beneficial bacterial flora is not cost-free. The symbiotic contract that has evolved between the bacterial flora and its host includes obligations—it requires that the host actively maintain intact mechanisms for properly managing the physiologic stresses that are closely associated with essential survival functions of the symbiotic bacteria. These stresses include the generation of oxygen free radicals in basic metabolic pathways and the production of proinflammatory bacterial structures, among others. The health of the host thus depends on preserving functional genes for handling peroxoxidative stress, bacterial antigens, and inflammation, as well as on the maintenance of an intact mucosal barrier.

The serious consequences of this contract between host and symbiont become evident in its breach. Heretofore considered quite benign, the normal bacterial flora has been shown in recent studies with knockout mice models to harbor latent tumorigenic potential. An imbalance in the host-symbiont relationship can trigger this hidden potential, to the detriment of the host. It is reasonable to suppose that similar pathways operate in the human body.

The normal bacterial flora is a prerequisite for the development of inflammation in the colon

Though most genetically-engineered mouse colon cancer models have not yet been tested under germ-free conditions, they present the common picture of a close association between inflammation and cancer development. There are several studies on the development of inflammation in these models under germ-free conditions (Table 2).

One of the best-characterized models in this respect is the IL-10-deficient mouse. Interleukin-10 (IL-10) affects the growth and differentiation of many hemopoietic cells in vitro; in particular, it is a potent suppressor of macrophage and T cell functions. IL-10-deficient mice showed increased morbidity and mortality when exposed to normal bacterial flora. All male IL-10-deficient mice housed under conventional conditions died by 4 mo, while 50% of females remained alive. In contrast, both male and female IL-10-KO mice were healthy when housed under germ-free conditions (up to 8 mo). Marked inflammation was observed in the conventional IL-10-deficient mice. Abnormal changes included: (1) thickening of the mucosa, (2) disorganization and hyperplasia of crypts, (3) epithelial erosion/ulceration, (4) accumulation of bacteria, (5) marked infiltration of leukocytes, and (6) crypt abscess formation. None of these inflammatory changes were found in the germ-free IL-10-deficient animals[13,14]. Neutrophil chemokine KC was produced by epithelial cells in response to the inflammatory mediators TNF-alpha and IFN-gamma that were expressed following exposure to normal flora in animals lacking IL-10[13]. Inducible nitric oxide synthase (iNOS) had no impact on the development or severity of spontaneous chronic inflammation in IL-10-deficient mice[15]. In contrast to other genetically-engineered rodents, germ-free IL-2-deficient mice were not free of inflammation. They developed mild inflammation, but remained clinically healthy during observation periods of up to 46 wk with no mortality[16]. In contrast, specific pathogen-free IL-2-deficient mice developed massive mononuclear infiltration with frequent crypt abscesses, and usually died between 28 and 32 wk of age. The enteric bacterial flora was also required for the development of inflammation in HLA-B27/β2m transgenic rats and TCR-deficient mice[17,18]. Mice deficient in the multiple drug resistance (mdr) gene (mdr1a) were susceptible to developing a severe, spontaneous intestinal inflammation when maintained under specific pathogen-free animal facility conditions. This model has not been tested under nonspecific germ-free conditions, but treating mdr1a-deficient mice with oral antibiotics both prevented the development of disease and resolved active inflammation, supporting the hypothesis that the enteric bacterial flora is required for colonic inflammation[19]. Similar to mdr1a-deficient mice, colitis in keratin 8-deficient mice was amenable to antibiotic therapy[20]. These studies indicate an essential role for the normal bacterial flora in the pathogenesis of inflammation.

Colonic inflammation could also be induced with Tgfβ1/Rag2, Smad3, Gαi2, or cytokeratin 8 gene knockouts[7,8,11,21], but the effect of the normal enteric bacterial flora on the development of inflammation has not been examined under germ-free conditions in these models (Table 2).

The bacterial flora as a whole is important in colonic inflammation and tumorigenesis

Abundant data have implicated intestinal bacteria in the initiation and amplification stages of inflammatory bowel diseases. However, the precise role of intestinal bacteria remains elusive. One theory is that both “protective” species and “harmful” species exist within the normal enteric bacterial flora. A healthy balance between these two populations in a normal host might be detrimental for an inflammation-prone host. Alternatively, a breakdown in the balance between the two populations, termed “dysbiosis”, could by itself promote inflammation in a normal host.

| Table 1  Effect of colonic bacterial flora on the development of gastrointestinal cancers in genetically-engineered mice |
| Gene                  | Function                        | Incidence of carcinoma (%) |
|-----------------------|---------------------------------|----------------------------|
| TCRβ/p53[β]           | Cellular immunity/              | 70                         |
|                       | growth and division             | 0                          |
| IL-10[γ]              | Anti-inflammation               | 7                          |
|                       |                                 | 0                          |
| Gpx1/Gpx2[β]          | Peroxidative stress            | 25                         |
|                       |                                 | 0                          |
| Gαi2[β]               | Cellular signaling             | 31                         |
|                       |                                 | ND                         |
| Smad3[γ]              | Tgfβ signaling                  | 100                        |
|                       |                                 | ND                         |
| Muc2[α]               | Major component of the mucus    | 69                         |
|                       |                                 | ND                         |
| Tgfβ1/Rag2[β,γ]       | Anti-inflammation/antigen        | 100                        |
|                       | receptor rearrangement          | ND                         |
| IL-2/β2m[γ]           | Proinflammation                 | 32                         |

ND: Not determined.
The pathogenic role of the normal enteric bacterial flora in the development of enterocolitis and colon cancer has been implicated in C57BL/6 IL-10-knockout mice. Probiotic Lactobacilli modify the enteric flora and are thought to have a beneficial effect on enterocolitis. Treatment of IL-10-deficient mice with the probiotic *Lactobacillus salivarius* ssp. *salivarius* UCC118 reduced the intensity of mucosal inflammation and the incidence of colon cancer from 50% to 10%. These effects were accompanied by significant reductions in fecal coliform, enterococci, and *Clostridium perfringens* levels. This study exemplifies the effect of changes at the flora level on the development of inflammation, and supports the hypothesis that there are “protective” species and “harmful” species in the normal bacterial flora.

Human studies using culture techniques have linked populations of *Bacteroides vulgatus*, *Eubacterium rectale*, *Ruminococcus albus*, *Peptostreptococcus productus*, *Bacteroides stercoris*, *Bifidobacterium angulatum*, *Eubacterium eligens*, *Ruminococcus gnavus*, *Fusobacterium prausnitzii*, *Eubacterium cylindroides* to a high risk of colon cancer. The fact that *Bifidobacterium longum* and *Bifidobacterium angulatum* and the total concentrations of bifidobacteria also are significantly associated with a high risk of colon cancer is contrary to suggestions that the ingestion of bifidobacteria cultures acts to increase the numbers of intestinal flora bacteria that might offer increased protection against colon cancer. [*Lactobacillus* S06 and *Eubacterium aerofaciens* and total lactobacillus concentrations were significantly associated with a low risk of colon cancer.]

Further studies of the population differences between normal and disease states might illuminate the contribution of specific flora changes to disease development.

**The bacterial flora potentiates tumor formation independent of inflammation**

Mutation of the APC gene leads to the development of multiple intestinal neoplasia (Min), especially in the small intestine, with little inflammation. Germ-free Min mice developed 50% fewer adenomas in the small intestine, although there were no significant differences in the remainder of the intestinal tract. This model suggests that commensal bacteria are not required, but can potentiates tumor formation independent of inflammation.

**Specific bacterial infection promotes colon tumor formation in genetically susceptible mice**

Although resident enteric bacteria are necessary for the development of spontaneous colitis in many rodent models, not all bacteria have an equivalent capability to induce inflammation. Germ-free IL-10-deficient mice populated with bacterial strains, including *Bacteroides vulgatus*, *Clostridium coryne*, *Streptococcus ruminianus*, *Escherichia coli*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus acidophilus*, *Lactobacillus lactis*, a *Bifidobacterium* sp., and a *Bacillus* sp., did not exhibit significant colitis. Conventional IL-10-deficient mice suffered from chronic enterocolitis. In contrast, mutants kept under specific pathogen-free conditions developed only a focal inflammation limited to the proximal colon. These results suggest that the bowel inflammation in the mutants was stimulated by single or multiple specific bacterial species. *Citrobacter rodentium*, *Helicobacter hepaticus*, *Enterococcus faecalis* are examples of conditional cancer-causing bacteria that alone do not cause cancer, but are carcinogenic in certain genetically-engineered mice.

**Citrobacter rodentium** is a gram-negative bacterium that colonizes predominantly the distal colon of mice, causing a disease termed transmissible murine colonic hyperplasia (TMCH). This condition induces colitis and crypt cell proliferation, similar to that seen in human idiopathic inflammatory bowel diseases, including Crohn's disease and ulcerative colitis. Infection with *C. rodentium* has not yet been associated with tumorigenesis, but TMCH promoted colon tumor development in mice administered the carcinogen DMH. *Apc* 

$^{min/1}$ mice infected with *C. rodentium* at 1 mo of age had a 4-fold increase in the number of colonadenomas at 6 mo of age, as compared with uninfected Min mice. *H. hepaticus* is a newly recognized bacterium associated with chronic active hepatitis, hepatic carcinoma, and inflammatory bowel disease in mice. *H. hepaticus* infection did not cause colon cancer in mice in the absence of an inflammatory trigger. Germ-free and specific pathogen-free TGFβ1/Rag2-deficient mice were free of inflammation, hyperplasia, and cancer, but when reintroduced into a *H. hepaticus*-containing specific
pathogen-free room, developed colonic adenoma/carcinoma\textsuperscript{11,38}. \textit{H. hepaticus} in pure culture did not seem to induce IBD in IL-10 KO mice\textsuperscript{39}, suggestive of an essential collaboration between \textit{H. hepaticus} and normal bacterial flora in the observed etiology.

\textit{Enterococcus faecalis}, previously known as group D \textit{Streptococcus} or \textit{Streptococcus faecalis}, is an opportunistic pathogen that is found in the alimentary tract of both humans and animals. Its notorious capacity to acquire virulence factors and antibiotic resistance genes have made this opportunistic bacterium a major problem for patients and clinicians\textsuperscript{40–42}. Inflammation, dysplasia, and rectal carcinoma developed in IL-10 KO mice colonized with \textit{E. faecalis}, but not in germ-free mice\textsuperscript{43}.

### Unusual bacterial infections associated with colorectal cancer in humans

Although there is no established bacterial pathogen for human colorectal cancer, unusual infections might precede the clinical diagnosis of cancer in some instances.

\textit{Streptococcus infantarius}, formally known as \textit{Streptococcus bovis} or Non-enterococcal Group D \textit{Streptococcus}, is a constituent of the human enteric flora. It is the causative agent in 5%-14% of endocarditis cases. Individuals with colon cancer had higher fecal carriage of \textit{S. bovis} as compared to patients with nonmalignant enteric disease and healthy controls\textsuperscript{44,45}. Panwalker reviewed 467 cases of adult \textit{Streptococcus bovis} bacteremia\textsuperscript{46}. Malignant colonic tumors were present in 62 of the 467 patients (13%). In some cases, the bacteremia will occur months or years after this species becomes established. It is not known whether the \textit{S. bovis} carriage has any direct cause-and-effect relation to colon cancer. In azoxymethane-treated rats, administration of either \textit{S. bovis} or its wall-extracted antigens promoted the progression of preneoplastic lesions through an increased formation of hyperproliferative aberrant colonic crypts, enhanced the expression of proliferation markers, and increased the production of IL-8 in the colonic mucosa, suggesting that \textit{S. bovis} acts as a promoter of early preneoplastic lesions in the rat colon. It is interesting that bacterial cell wall proteins were more potent inducers of neoplastic transformation than were the intact bacteria\textsuperscript{47}. When a cell wall protein fraction composed of 12 different proteins was applied to human epithelial colonic Caco-2 cells or the rat colonic mucosa, it was able to trigger the release of CXC chemokines (human IL-8 or rat CINC/GRO) and prostaglandin E2, which are correlated with an \textit{in vitro} over-expression of COX-2. Moreover, these proteins were highly effective in the promotion of preneoplastic lesions in azoxymethane-treated rats. In the presence of these proteins, Caco-2 cells exhibited enhanced phosphorylation of MAP kinases. These data suggest that bacterial components possess both proinflammatory properties and procarcinogenic potentials, and support the hypothesis that colonic bacteria can contribute to cancer development, particularly in association with chronic infection/inflammation diseases where these bacterial components might interfere with cell function\textsuperscript{48}.

\textit{Clostridium septicum} infections are rare, but are often associated with malignancy. This association has been discussed in a number of case reports\textsuperscript{12,49}. In a review of 162 published cases of \textit{C. septicum} infection in 1989, 81% had an associated malignancy, 34% had an associated colon carcinoma, while 40% had an associated hematologic malignancy\textsuperscript{13}. A consequence of these close associations is the cost of patients for whom a \textit{C. septicum} infection is diagnosed, a rigorous search for occult malignancy should be mounted. It is yet to be determined whether these associations represent a cause-and-effect relationship or a secondary phenomenon.

For both \textit{S. bovis} and \textit{C. septicum}, a question remains whether the bacteria induce bowel cancer or if the growth of the organisms is promoted by a preexisting carcinoma. Testing their carcinogenicity in genetically-engineered immunodeficient mouse models might shed light on our understanding of the role of bacteria in the development of human cancers.

### SUMMARY AND PERSPECTIVE

Traditional bacteriology is built upon concepts developed from studies of infectious diseases in which a pathogen can often be identified and pathogenesis explained by toxins or virulent factors. These concepts have clearly demonstrated their usefulness in the identification of etiologic agents of anthrax in the nineteenth century and \textit{Helicobacter} gastritis more recently, among many others. Recent studies of tumorigenesis in rodent models have opened a new chapter in bacteriology, with the observation that the normal bacterial flora actively participates in the development of cancers. Searching for the responsible agents among normal flora bacteria, albeit not classic pathogens, is still a reasonable approach in the assessment of bacterial roles in chronic inflammatory disorders and related cancers. Support for this approach is found in evidence that certain bacterial species are required for tumorigenesis in genetically deficient rodent models, and in the association of specific bacterial species with human cancers.

The capacity for causing inflammation or cancers that is exhibited by a complex bacterial flora might depend on the aggregate activity of multiple constituents of the flora, rather than on a single species. This capacity might be enhanced or reduced as a function of significant changes in the species diversity and abundance within the flora. Thus, more detailed knowledge of how the bacterial flora is altered in association with disease conditions could significantly broaden our understanding of the etiology and pathogenesis of these diseases. In the past, the connection between a heterogeneous bacterial flora and a disease was often regarded as too complex to interpret. However, recent advances in 16S rDNA technology, DNA sequencing, and data analysis tools have made it possible to determine the differences between two diverse bacterial flora by defining and comparing the majority of bacterial species and their prevalence in each flora\textsuperscript{14,49,50}.

Alternatively, the host might also play a decisive role here. Colon cancers might develop in a genetically deficient host in the presence of a normal bacterial flora, without any requirement for specific bacterial species or alteration of the flora. No human counterparts of the critical gene...
defects artificially created in rodents have been identified, but it is plausible that weak defects or polymorphisms of human genes that might not be aberrant enough to confer an overt clinical disease could, in collaboration with a normal bacterial flora, lead to cancers in a patient over a period of decades. Future investigations could seek to discover any relevant weak gene defects in human patients, and correlate them with long-term pathogenesis.

The present studies in rodent models of the role of the bacterial flora in tumorigenesis obviously raise some interesting possibilities in relation to human cancers. Can the concepts derived from bacterial flora in colon cancers be extrapolated to cancers in other anatomic sites where cancer development is related to chronic inflammation, such as gastric adenocarcinoma in H pylori gastritis, esophageal adenocarcinoma in reflux esophagitis, or oral squamous cell carcinoma in periodontitis? Can the natural history of cancer development be altered toward the benefit of hosts by manipulation of bacterial flora using probiotic and antibiotic therapies or vaccination? The potential contributions to improving human health make finding the answers to these questions a clear priority for future research.

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