Review

Severe Defects in the Macrophage Barrier to Gut Microflora in Inflammatory Bowel Disease and Colon Cancer

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Abstract. *The continuity of the subepithelial band of lamina propria-indigenous macrophages (SBLP-M) discourages commensal gut bacteria from invading the host. In this Review we analyzed the impact of a disintegrating SBLP-M in inflammatory bowel disease (IBD), which results in microbiota inflow, inadequate immune responses and IBD-associated colon cancer. In previous work, we analyzed endoscopic biopsies taken from normal-looking descending colon in 247 patients with IBD, and 167 from control patients without IBD. Sections immunostained for cluster of differentiation 68 (CD68) protein showed no inflammatory changes. In IBD, the band of CD68+ SBLP-M was fragmented or minute in 59% (47/80) and absent in 9% (7/80). In contrast, only 31% (51/167) of the biopsies from control patients had a fragmented/minute band of CD68+ SBLP-M and this band was not absent in any ($p < 0.05$). The finding that the band of CD68+ SBLP-M was fragmented to totally lost in IBD suggests a long-lasting defect in the barrier against the gut microbiome in IBD. The lack of ongoing inflammation in colonic biopsies should rule-out the participation of bone marrow-derived inflammation-elicited macrophages in loss of the barrier. Today, it is widely accepted that dysbiosis and inappropriate immune response to microbial flora play a pivotal role in the pathogenesis and development of IBD-associated colon cancer. Based on present knowledge, it is submitted that defects in the SBLP-M barrier in IBD encourage the trespassing*

of the gut microflora into the host, thereby destabilizing host immunity. These events in concert may play the ultimate pivotal role in the evolution of colon cancer in patients with IBD.

In recent years, much concern has been raised over the role played by the gut microbiome and gut inflammation, and their effect on the host immune system and colonic carcinogenesis. The cross-talk between the gut commensal microbiota and the subepithelial resident (indigenous) macrophages encourages the creation of a protective barrier of macrophages. Under normal conditions, an unbroken barrier of colonic macrophages is essential in hampering host invasion by commensal bacteria. The mechanisms whereby the gut bacteriome reaches the host remains unclear. In this review, we discuss the possible impact of a defective macrophage barrier in the gut on the putative trespassing of the gut bacteriome in inflammatory bowel disease (IBD), their effect on the host immune system and the development of IBD-associated colonic carcinoma.

Macrophages in the Colonic Mucosa

The mononuclear phagocytic system is composed of blood leucocytes (monocytes and macrophage), tissue macrophages (histiocytes), and Kupffer cells. In this superfamily, two macrophage systems are recognized, one derived from bone-marrow lineage-committed monocytes (BMMs), the other of resident indigenous macrophages (1, 2). BMMs circulate in the blood, but when chemo-attractants are released from tissues injured by trauma or by infections, BMMs leave the circulation. In the chemically different hostile microenvironment, BMMs differentiate into macrophages. Of the resident indigenous macrophages, the gut macrophages comprise the largest macrophage pool in the body (3). In the non-inflamed mucosa, gut macrophages are inflammation anergic, but retain scavenger and host defense function, a perfect attribute for macrophages in close proximity to gut microbiota.

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Submucosal Macrophages in IBD

Under normal conditions, indigenous gut macrophages gather as a continuous band in the subepithelial *lamina propria* (SBLP) of the colon (4). SBLP macrophages are crucial in deterring commensal gut microbiota from attacking their host by rapidly eliminating gut microflora (5-7). By producing over 100 secretory products, gut SBLP macrophages choreograph pro-inflammatory and host defense activities (8, 9). Recognition receptors allow SBLP macrophages to detect, engulf and kill a complex and vast number of different bacteria, bacteriophages, fungi, and viruses (gut virome), collectively called gut microbiota (10, 11). During an inflammatory response, both resident gut SBLP macrophages and inflammation-elicited macrophages, derived from blood-circulating monocytes, play crucial roles in the control of infection by actively pursue invading microorganisms, through degradation and release of inflammatory mediators (7).

Breakdown of the SBLP Macrophages in IBD

A breakdown of the integrity of SBLP macrophages in IBD allow both alien and commensal gut bacteria to trespass that barrier, resulting in host invasion, thereby altering natural host immunity. In previous work, we analyzed the characteristics of the band of CD68+ SBLP macrophages in colonic biopsies without ongoing inflammation (to exclude the participation of inflammation-elicited macrophages) in patients with protracted IBD (12). Left colon biopsies without ongoing inflammation harvested from 247 patients with endoscopically normal left colon were reviewed. Eighty were from patients with IBD (27 had ulcerative colitis in remission and 53 had right-sided Crohn's colitis). The remaining 167 were non-IBD control patients (90 had chronic colonic diarrhea, 63 were enrolled in a surveillance colorectal cancer (CRC) program, seven had microscopic colitis (four collagenous and three lymphocytic), and seven had miscellaneous colonic conditions (two infectious colitis, two HIV-associated colitis, one Bechterew colitis and two melanosis coli). Sections were stained with hematoxylin and eosin and immunostained for cluster of differentiation 68 protein (CD68). CD68 is a glycosylated lysosomal membrane protein expressed in the cytoplasmic granules of cells with phagocytic properties. The pattern of CD68+ macrophages found in the SBLP were divided into four types: i) Continuous, when an unbroken band of CD68+ macrophages was found for the entire section; ii) fragmented, when one or more segments of that band were missing from the entire section; iii) minute, when only five or more CD68+ macrophages were found in the entire section; and iv) absent when ≤ 4 consecutive CD68+ macrophages were found in the entire section. In IBD, the

band of SBLP CD68+ macrophages was fragmented or minute in 59% (47/80) and negative in 9% (7/80). In contrast, only 31% (51/167) of the control patients had a fragmented/minute band, and a band was absent from none. The difference in the continuity of the CD68+ macrophage band between IBD and controls was significant ($p < 0.05$).

The cross-talk between the gut commensal microbiota and the gut CD68+ SBLP macrophages results in the formation of a barrier of SBLP macrophages (12). This band is the first line of defense (gatekeeper) against gut-microbiota intrusion deep into the LP (4, 12). Before reaching the LP, the microbiota must successively overcome several obstacles: i) The thick mucous layer covering the epithelial surface (13); ii) the natural tight junctions that intertwine colonic epithelial cells (14); iii) the natural peptide family of trefoil factors 1, 2, and 3 (15); and the innate antimicrobial enzyme lysozyme (16-18). Thus, a breach of the natural continuous SBLP macrophage barrier only arises after the other aforementioned barriers have been overcome. When all these barriers have successively been penetrated, dendritic cells (19) and remnant deeper LP macrophages would be responsible for counteracting invading microbiota. Hence, the proportion of cases with a fragmented/minute or even lacking a CD68+ SBLP macrophage band was significantly higher in patients with IBD than in controls, suggesting a long-lasting lack in continuity of the CD68+ SBLP macrophage barrier against the altered IBD-associated gut microbiome. The lack of ongoing inflammation in colonic biopsies ruled-out the participation of bone marrow-derived inflammation-elicited macrophages in the loss of the SBLP macrophage band.

The Normal Colonic Bacterial Flora

Healthy gut microbiomes as assessed by sequencing are consistently dominated by bacteria of two phyla, Bacteroidetes and Firmicutes (20). A core set of more than 50 distinct bacterial genera is found, among them, *Enterobacteria*, *Enterococci*, *Lactobacilli*, *Clostridia*, *Bacteroides*, gram-positive non-sporing anaerobes (*Eubacteria* and *Bifidobacteria*) (21). Over 1,000 bacterial species of the gut have now been characterized, providing a significant inventory of bacterial constituents. Interestingly, molecular phylogenetics has led to the reclassification of many of these species in the past 20 years (20). Of particular interest, species within *Bacteroides*, previously considered the most prevalent and abundant bacterial genus in the gut, have been reclassified into five genera: *Alistipes*, *Prevotella*, *Paraprevotella*, *Parabacteroides*, and *Odoribacter* (22).

Compiled data from these studies (22) identified 2,172 species isolated from human beings, classified into 12 different phyla, of which 93.5% belonged to Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes. Three of the 12 identified phyla included only one species isolated from humans, namely

an intestinal species, *Akkermansia muciniphila*, the only known representative of the Verrucomicrobia phylum. In humans, 386 of the identified species are strictly anaerobic and will therefore generally be found in mucosal regions such as the oral cavity and the gastrointestinal tract (22).

Altered Bacteriome in Sporadic Colonic Cancer

Tilg *et al.* recently reviewed the important role of *Fusobacterium nucleatum*, *Escherichia coli*, and *Bacteroides fragilis* in sporadic colon cancer (23). The intestinal microbiota also comprises viruses and fungi, but the role of viruses and fungi in CRC has not been investigated. Rubinstein *et al.* demonstrated that *F. nucleatum*, through *Fusobacterium* adhesin A (FadA), which binds to E-cadherin, thereby activating the β -catenin signaling pathway, resulting in induction of oncogenic and inflammatory responses (24). Importantly, FadA gene expression in human CRC tissue was found to be extraordinarily high compared with healthy controls, *F. nucleatum* increased tumor multiplicity in the adenomatous polyposis coli/multiple intestinal neoplasia (ApcMin/+) model of intestinal tumorigenesis (25). *E. coli* is a gut commensal, although certain strains have acquired the ability to promote intestinal inflammation and to produce toxins such as colibactin with oncogenic potential (26). Colonization of a colon cancer-associated *E. coli* strain in ApcMin/+ mice resulted in a marked increase in the number of polyps, suggesting that certain *E. coli* strains might indeed promote tumorigenesis. Similarly to *E. coli*, largely experimental evidence supports a role for *B. fragilis* in intestinal tumorigenesis. *B. fragilis* comprises about 1-2% of the commensal microbiota in most humans.

It should be mentioned that the colonic microbiota also includes viruses and fungi. Nonetheless, the role of viruses and fungi in colon carcinogenesis remains unclear (27).

Altered Bacteriome in IBD

An altered gut microbiota has repeatedly been found in patients with IBD (28-34). The concept of an altered gut microbiota, called dysbiosis (epitomized by the expansion of bacterial taxa), is possibly the most significant development in IBD research in the past decade. In recent years, the types of bacteria found in IBD have intensively been investigated. Lower levels of *B. bifidobacterium*, *Faecali bacterium*, *Saccharomyces cerevisiae* and *Lactobacillus* are found but increased proportions of *Basidiomycota/Ascomycota*, and levels of *Proteobacteria*, *Fusobacterium* spp, *E. faecalis*, *Neisseriaceae blauti*, *Candida albicans*, *Haemophilus influenzae* and other *Haemophilus* species, and aggressive adherent invasive *E. coli* pathovar have been found (28-34). In addition, Kostic *et al.* reported a decrease in alpha diversity, *Clostridia*, *Ruminococcaceae erysipelotrichales*,

Bacteroidales and *Clostridiales* in IBD (29), whereas Gevers *et al.* identified a strong correlation of Crohn's disease with increased abundance of *Enterobacteriaceae*, *Pasteurellaceae*, *Veillonellaceae*, and *Fusobacteriaceae*, and a decreased abundance of *E. coli* was also found within intestinal macrophages (34).

Altered Bacteriome in IBD-associated Colon Cancer

The defects in the SBLP macrophage barrier in IBD would allow trespassing of the indigenous and of the alien microflora into the host, an anomaly that alters natural host immunity. Today, it is widely accepted that dysbiosis and inappropriate immune response to microbial flora play a pivotal role in the pathogenesis and development of IBD-associated CRC (35-39). These findings have been substantiated in experimental animals (23, 40-42). A study by Wu *et al.* revealed that enterotoxigenic *B. bifidobacterium*, which secretes the toxin BFT, induces colitis and colonic tumors in ApcMin/+ mice (43). Detection of certain bacterial strains, namely the combination of *F. nucleatum*, *Bacteroides clarus*, *Roseburia intestinalis*, and *Clostridium hathewayi*, demonstrated a sensitivity >90% and a specificity >80% for CRC (44). Colonic polyposis in Apc^{Min/+} mice is accompanied by accumulation of microbes in polyps, triggering local inflammatory responses. Native Americans with a low CRC risk exhibit lower stool levels of total short-chain fatty acid butyrate compared with African/Caucasian Americans having a considerably higher CRC risk (45). It is likely that a combination of alterations, rather than increased or decreased abundance of a particular strain in the intestinal microbiota promotes tumorigenesis. Remarkably, patients with Crohn's disease exhibit similar microbial alterations (*e.g.*, increased abundance of *E. coli* and *F. nucleatum*) as seen in CRC (46), suggesting not only inflammation but also a shared dysbiosis may contribute to IBD-associated CRC (23).

Host Immunity and Colon Cancer

There is growing evidence that the host immune system critically controls intestinal carcinogenesis (28, 47). Accumulating evidence indicates that intestinal microflora has protective, metabolic, trophic and immunological functions and are able to establish cross-talk with the immune component of mucosal immunity, comprising cellular and soluble elements. Microbiota communicate with the immune system through toll-like receptor signaling (48) and inflammasome sensing nucleotide-binding oligomerization domain (NOD)-like receptors (49) have been implicated in the progression of colonic tumorigenesis. NOD1 recognizes bacterial antigens, which triggers an immune response, and NOD1 deficiency resulted in increased development of tumors in Apc^{Min/+} mice and in an inflammation-related tumor model (50).

Conclusion

Today, it is widely accepted that dysbiosis and inappropriate immune response to microbial flora play a pivotal role in the pathogenesis and development of IBD-associated colon cancer. Based on present knowledge, it is submitted that defects in the SBLP-macrophage barrier in IBD encourages the trespassing of the gut microflora into the host, thereby destabilizing host immunity. These events in concert play a pivotal role in the evolution of colon cancer in patients with IBD.

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