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Intestinal Host-Microbiome Interactions

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1. Introduction

A human body contains at least tenfold more bacteria cells than human cells and the most abundant and diverse microbial community (also known as microbiota or microbiome) resides in the large intestine (colon). It is estimated that this colonic microbiome is composed of $\sim 10^{14}$ bacterial cells, comprising $>10^3$ species (Dethlefsen *et al.*, 2006; Qin *et al.*, 2010). Intestinal microbiomes differ from individual to individual but remain relatively stable during adult life (Green *et al.*, 2006; Arumugam *et al.*, 2011). The resident microbiome provides the host with core functions that are essential for digestion of food and control of intestinal epithelial homeostasis. Conversely, an increasing body of evidence supports a relationship between infective agents and human colorectal cancer (CRC) by production of DNA damaging metabolites or toxins, and the induction of cell proliferation and pro-carcinogenesis pathways by a subpopulation of the intestinal microbiota. It could be speculated that the intrinsic intestinal microbiome of a certain individual may contain an unfavorable number of disease-inducing bacteria. On the long term, their activities may override the health-promoting activities of the commensal bacterial population. On the other hand, the dramatic physiological alterations that result from colon carcinogenesis itself (Hirayama *et al.*, 2009) disturbs the local intestinal microenvironment and causes (local) shifts in the microbiota composition and provides a portal of infection for certain opportunistic pathogens. The latter phenomenon could explain why some uncommon bacterial infections are often associated with CRC. In this chapter we will discuss the mechanisms by which intestinal bacteria may drive the initiation and progression of sporadic CRC, but also the driving forces of intestinal carcinogenesis on local microbial dysbiosis and the consequences thereof will be reviewed.

2. Intestinal microbiome

The colonic epithelium is the first line of defense against enteric antigens and bacteria. In a healthy colon, the epithelial barrier regulates uptake of nutrients and limits uptake of potential toxic substances and infectious agents (Chichlowski & Hale, 2008). Goblet cells are specialized epithelial cells within the mucosa that produce a viscous mucus layer that covers the intestinal epithelium (Heazlewood *et al.*, 2008). This mucus layer is thick and consists of an inner firmly attached layer, that excludes bacteria from direct contact with the

underlying mucosa, and an outer loose mucus layer that mainly functions as lubricant (Atuma *et al.*, 2001). Bacterial colonization of the gastrointestinal tract occurs during the first two years of life. After this period, the microbiota composition is rather stable throughout adulthood (Dethlefsen *et al.*, 2006). Nevertheless, it is likely that the colonic microbiota transiently respond to dietary intake and host physiology (Thompson-Chagoyan *et al.*, 2007). The inter-individual microbiomes differ consistently, however, it is thought that these different marked microbiota may perform similar functions, and genetically complement their host with crucial physiological functions that are not provided by the human genome itself (Candela *et al.* 2010; Gill *et al.*, 2006; Neish, 2009; O'Hara & Shanahan, 2006; Xu *et al.*, 2007). Intestinal microbiome-specific metabolic functions increase energy yield and storage from diet, regulate fat storage and generate essential vitamins, which are primarily due to the fermentation of indigestible dietary polysaccharides (Neish, 2009). It has been shown that mucosa-associated bacteria differ from the community recovered from feces, but are rather uniformly distributed throughout the colon (Green *et al.*, 2006; Macfarlane *et al.*, 2004; Zoetendal *et al.*, 2002). This mucosa-adherent population is less prone to physiological effects, such as dietary changes (Sonnenburg *et al.*, 2004), and prohibits colonization of intruding pathogens (Stecher & Hardt, 2008). Malfunctioning of the host epithelial defense mechanisms, increases the risk for bacterial infection and intestinal inflammation, as seen in patients with inflammatory bowel disease (IBD). Intestinal disease can also be directly triggered by enteropathogenic pathogens, like *Shigella*, *Citrobacter* and *Salmonella* species, that avail of virulence mechanisms that allows them to outcompete the commensal mucosa-associated bacterial population and to breach the mucosal barrier and intestinal innate immune system (Stecher *et al.*, 2007).

3. Bacterial promotion of CRC

The genetic background of the host together with dietary intake, influences the microbial composition in the gut. However progression of CRC itself also influences the gut barrier and micro-environment in the intestine. This dynamic interplay between environment, genetic and microbial influences makes it hard to dissect the exact contribution of the microbiota in the development and progression of CRC. In the next paragraphs, the mechanisms by which the intestinal microbiota could contribute to CRC are further discussed. The significance of the intestinal microbiome on the development of CRC is probably best illustrated by the fact that patients with IBD, which originates from an altered host response to a normal intestinal bacterial population (Round & Mazmanian, 2009), have a high predisposition for CRC (Macfarlane *et al.*, 2005).

3.1 Promotion of tumorigenesis

The effect of intestinal bacteria on CRC development has been studied in the intestinal neoplasia mouse model (*Apc^{min/+}*). This mutant mouse strain carries a heterozygous mutation in the *APC* locus (Moser *et al.*, 1990), meaning that only a single hit in the wild-type allele results in adenoma formation. Studies with germ-free *Apc^{min/+}* mice revealed that the formation of adenomas was strongly reduced by as much as 50%, compared to mice bred under conventional conditions (Dove *et al.*, 1997; Moser *et al.*, 1990; Su *et al.*, 1992). When such mice were exposed to enterotoxigenic *Bacteroides fragilis* (ETBF), tumors developed more rapidly, whereas mice colonized with non-toxicogenic *Bacteroides fragilis*

(NTBF) showed no increased tumor formation compared to conventional mice (Housseau & Sears, 2010).

These data clearly show that the intestinal microbial population has a strong promoting effect on tumor progression in mice that have a genetic predisposition for developing intestinal adenomas and that certain species within the intestinal microbiota contribute more than average to this process.

3.2 Stimulation of TLR signaling

A balanced immune stimulation to commensal and pathogenic bacteria is crucial for a healthy intestinal tract. Toll-like receptors (TLRs) are proteins that activate immune responses towards potentially harmful pathogens upon sensing of pathogenic substances, such as cell wall components. However, chronic overstimulation of these responses may be detrimental by leading to the initiation and progression of CRC (Fukata & Abreu, 2007).

A direct impact of bacteria on the development of CRC through the TLR5/MyD88 pathway was demonstrated in germ-free and gnotobiotic mice. These animal experiments revealed that *MyD88*^{-/-} knock-out mice that were treated with the carcinogen azoxymethane (AOM) failed to develop colorectal tumors when these mice were subjected to bacteria. In contrast, control mice rapidly developed CRC upon bacterial colonization of their intestinal tract. These results implicate that TLR/MyD88 signaling is a prerequisite for the development of CRC (Uronis *et al.*, 2009). In addition, it was shown that tumors in *Apc*^{min/+} *MyD88*^{-/-} mice were significantly smaller than those found in *Apc*^{min/+} mice (Rakoff-Nahoum & Medzhitov, 2007). Another study showed that *TLR4*^{-/-} mice were partly protected against the development of neoplasia by tumor-inducing chemical agents (Killeen *et al.*, 2009). Additional evidence was presented that TLR4 signaling can promote colon carcinogenesis by stimulating tumor infiltration of Th17 cells (T-helper cell subset that produces IL-17) through the increased production of pro-inflammatory signals (Su *et al.*, 2010). It can be envisaged that bacterial TLR4 ligands, such as LPS, play an important role in this increased chemotactic activity of tumor cells (Scanlan *et al.*, 2008). Importantly, Th17 cells have directly been implicated in the pathogenesis of Enterotoxigenic *Bacteroides fragilis*-induced CRC (Housseau & Sears, 2010; Wu *et al.*, 2009). Thus, although TLR signaling is important for the effective clearance of harmful pathogens and can mediate anti-tumor cell responses, chronic TLR activation may tip the delicate balance towards tumor-promoting activities (Rakoff-Nahoum & Medzhitov, 2009).

Altogether, the above mentioned studies indicate that chronic bacterial stimulation of inflammatory pathways at malignant sites promotes, and may even be a prerequisite for, intestinal tumor development.

3.3 Upregulation of COX-2

Cyclooxygenase-2 (COX-2) is one of the key players in the progression of CRC. The expression of COX-2 is highly elevated in colonic tumors and correlated with disease stage and stimulates cell proliferation and pro-inflammatory pathways by the production of prostaglandins (Menter *et al.*, 2010). Human intervention studies have clearly shown that the usage of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) can reduce CRC risk by as much as 75% (Eaden *et al.*, 2000; Labayle *et al.*, 1991; Thun *et al.*, 1991). Evidence for bacterial involvement in the upregulation of COX-2 during CRC development was gained through animal and *in vitro* studies. First, superoxide radicals produced by *Enterococcus faecalis* were

shown to upregulate the expression of COX-2 in hybrid hamster cells containing human chromosomes, as well as in macrophages (Wang & Huycke, 2007). Furthermore, macrophages that were pre-treated with a COX-2 inhibitor and subsequently exposed to *E. faecalis* totally inhibited the induction of chromosome instability (CIN) in these hybrid hamster cells. Second, an animal study published by Ellmerich *et al.* (2000b) indicated that *Streptococcus bovis* biotype II.1 (*Streptococcus infantarius*) could also play a role in the progression of CRC through induction of the COX-2 pathway. These investigators employed a rat model in which pre-treatment with azoxymethane (AOM) induced pre-neoplastic aberrant crypt foci (ACF). When such rats were co-exposed to *S. infantarius* or cell wall antigens from this bacterium, the number of ACF increased drastically and also adenomas were found, whereas the latter were totally absent in the control mice treated with AOM alone. In addition, the production of the pro-inflammatory cytokine IL-8 in the mucosa of rats exposed to *S. infantarius* was increased. This finding is in accordance with *in vitro* studies on epithelial Caco-2 cells that release both IL-8 and PGE2 upon incubation with *S. infantarius* (Biaric *et al.*, 2004). Moreover, Abdulmir *et al.* (2010) have recently shown that increased COX-2 and IL-8 expression was associated with the presence of *Streptococcus gallolyticus* (*S. bovis* biotype I) in human colon tumor tissue. However, IL-8 expression was not increased in non-malignant tissue that contained *S. gallolyticus*. Together these studies indicate that COX-2 induction is associated with both tumor development and exposure to bacterial stimulants.

3.4 Toxin-induced promotion of cell proliferation

Enterotoxigenic *Bacteroides fragilis* (ETBF) has been implicated in the promotion of CRC through inflammatory pathways. *B. fragilis* is a normal inhabitant of the gastrointestinal tract, but its enterotoxigenic form is only present in approximately 20% of the healthy population (Sears, 2009). ETBF produces the *B. fragilis* toxin that degrades E-cadherin in epithelial cells, which causes β -catenin to migrate towards the nucleus where it can activate cell proliferation pathways (Wu *et al.*, 2003). Consequently, *APC*^{min/+} mice colonized with ETBF were shown to suffer from increased tumor burden compared to control mice colonized with non-toxigenic *B. fragilis* (NTBF) strains (Housseau & Sears, 2010; Wu *et al.*, 2009). Importantly, Wu *et al.* (2009) showed that this increased tumor burden was mediated through the increased expression of STAT3 that leads to a Th17 response. Importantly, increased tumor formation could be blocked by anti-IL17 therapy. These experiments clearly show that induction of a STAT3/Th17-dependent pathway for inflammation, leads to inflammation-induced cancer by ETBF in a mouse model. Since ETBF is a quite common bacterium in the gastro-intestinal tract, this finding could have major implications for the role of these bacteria in the development of CRC in the human population. This idea is further corroborated by the fact that patients with CRC have indeed increased carriage rates of ETBF compared to NTBF (Toprak *et al.*, 2006). It should be realized that this mechanism of tumor induction could also be associated with other toxigenic intestinal bacterial strains.

3.5 Toxin-induced DNA damage

Certain *E. coli* strains can induce increased mutation rates in eukaryotic cells as demonstrated by Cuevas-Ramos and colleagues (2010). Their experiments showed that *E. coli* strains harboring the *pks* island caused DNA damage in human epithelial cells and in an

ex vivo mouse intestinal model by the induction of single strand breaks and activation of DNA damage signaling pathways. The *pks* gene cluster codes for nonribosomal peptide synthetases and polyketide synthetases (*pks*) that synthesize a genotoxin named Colibactin. The *pks* island is commonly present in about 34% of commensal *E. coli* isolates. Upon infection of epithelial cells with physiological concentrations of *pks*⁺ strains, initial DNA damage occurred. Furthermore, it was shown that cells continued to proliferate in the presence of DNA damage after *E. coli* infection, resulting in an increased mutation frequency (Cuevas-Ramos *et al.*, 2010). These studies suggest that *pks*⁺ strains of *E. coli* could be involved in the initiation and progression of CRC. As, *E. coli* is generally regarded as a normal commensal inhabitant of the gastro-intestinal tract, Bronowski and co-workers investigated the differences between *E. coli* strains collected from healthy individuals and CRC patients (Bronowski *et al.*, 2008). These experiments showed that a subset of *E. coli* strains recovered from CRC tissue shared pathogenicity islands, encoding an alpha haemolysin and a cytotoxic necrotizing factor, with uropathogenic *E. coli* strains. This suggests that besides Colibactin production, other virulence characteristics may also mediate the tumor promoting capacity of *E. coli pks*⁺ strains.

3.6 Metabolite-induced DNA damage

Sulfate reducing bacteria use sulfate as energy source by converting it to sulfide and hydrogen sulfide (H₂S) in the human colon. The genotoxic potential of H₂S is in part mediated by oxidative free radicals, which results in increased levels of DNA damage in cultured epithelial cells (Attene-Ramos *et al.*, 2006; Attene-Ramos *et al.*, 2007; Attene-Ramos *et al.*, 2010). Furthermore, exposure to H₂S may disrupt the balance between apoptosis, proliferation and differentiation (Cai *et al.*, 2010; Deplancke & Gaskins, 2003). Interestingly, also COX-2 was shown to be upregulated in epithelial cells after H₂S treatment at physiological concentrations, probably through generation of reactive oxygen species (Attene-Ramos *et al.*, 2010). Increased fecal H₂S concentration was implicated as a risk factor for the development of colonic neoplasia in a clinical study (Kanazawa *et al.*, 1996). Whether these increased H₂S levels originates from increased activity of sulfate reducing bacteria and/or reduced epithelial capacity to degrade H₂S remains to be investigated.

E. faecalis was also found to produce extracellular superoxide in colonic tissue of rats, which is the result of dysfunctional microbial respiration (Huycke *et al.*, 2002). These rats produced up to 25-fold increased concentrations of hydroxylated aromatic metabolites in urine than rats colonized with a closely-related strain. Importantly, superoxide can be converted to hydrogen peroxide, which has the potential to diffuse into epithelial cells and cause DNA damage. In an *in vitro* setup, it was shown that the formation of DNA adducts by *E. faecalis* was mediated by activated COX-2 expression in macrophages that in turn promoted DNA damage in epithelial target cells (Wang & Huycke, 2007; Wang *et al.*, 2008). Since COX-2 induction has a clear clinical association with CRC, this might indicate that superoxide-producing bacteria have a contributing role in disease development. This notion is further underscored by the finding that *E. faecalis* fecal carriage was increased in CRC patients, whereas the number of butyrate producing bacteria was decreased (Balamurugan *et al.*, 2008). However, no clinical evidence has been presented that associates superoxide producing enterococci with adenomas or CRC (Winters *et al.*, 1998). This clearly indicates that, although the *in vitro* data and animal studies strongly suggest that oxygen radicals from bacterial origin could play an important role in CRC initiation or progression, the

clinical impact of these findings remains to be properly examined in well-designed clinical studies (Huycke & Gaskins, 2004).

Bacteroides species produce fecapentaenes that are potent mutagens that have been shown to alkylate DNA, which leads to mutagenic adducts. Some evidence points towards a mechanism in which oxygen radicals cause oxidative damage to DNA (Hinzman *et al.*, 1987; Povey *et al.*, 1991; Shioya *et al.*, 1989). Fecapentaenes appear in relatively high concentrations in human feces, however, no significant differences in fecapentaene levels were found in feces from CRC patients and controls (Schiffman *et al.*, 1989). In view of their mutagenic potential, however, fecapentaenes should be regarded as possible bacterial inducers of CRC (de Kok & van Maanen, 2000). For instance, their detrimental effects may locally contribute to the accumulation of mutations in epithelial cells, which is not directly reflected by the increased levels in fecal material.

3.7 Induction of pro-carcinogenic pathways

Some evidence exists that certain intestinal bacteria can also directly induce host epithelial pathways that make cells more susceptible to DNA damage by carcinogenic substances. Maddocks *et al.* (2009) have shown that enteropathogenic *E. coli* can down-regulate mismatch repair genes in colon epithelial cells. It may be envisaged that this impaired expression can lead to a net increased mutation rate upon co-exposure to genotoxic dietary compounds. This study accentuates that bacteria can directly interfere with gene expression in epithelial cells which, under certain conditions, may lead to increased carcinogenesis rates.

4. CRC microbiome

The preceding paragraphs describe the potential mechanisms by which bacteria can play a role in the initiation and progression of CRC. In the following paragraphs, the effects of colonic malignancies on the (local) microbial composition are discussed. It is evident that the dramatic physiological and metabolic alterations that result from colon carcinogenesis itself (Hirayama *et al.*, 2009) will locally disturb the intestinal environment. Consequently, this will cause (local) shifts in microbiota composition as the altered tumor metabolites and intestinal physiology will recruit a bacterial population with a competitive advantage in this specific microenvironment. This is exemplified by the fact that infections with certain opportunistic intestinal pathogens have been associated with CRC for many years (see Section 5). Thus pre-malignant sites seem to constitute a preferred niche for a subset of intestinal bacteria and facilitate their outgrowth and eventually entry into the human body. Importantly, local outgrowth of harmful bacteria could also accelerate tumor progression after disease has been initiated by other factors.

The effect of colonic tumors on the microbiome composition has been investigated by several studies. First, Scanlan *et al.* (2008) investigated the bacterial diversity in healthy, polypectomized patients with increased risk for CRC and CRC patients. These studies showed a significant increased diversity of the *Clostridium leptum* and coccoides subgroups in the CRC patients compared to a healthy control group. Importantly, metabonomic faecal water analysis was able to distinguish CRC and polypectomized patients from healthy individuals, which is indicative for an altered metabolic activity of the intestinal microbiota

in these patients. In another study by Maddocks *et al.* (2009) it was shown that the mucosa of adenomas and carcinomas contained increased numbers of *E. coli* compared to colonic mucosa from healthy controls. It was speculated that certain surface antigens on tumor cells, which display homology to surface antigens of fetal origin, may be responsible for the binding of *E. coli* and thus local recruitment of these bacterial strains (Martin *et al.*, 2004; Maddocks *et al.*, 2009; Swidsinski *et al.*, 1998). A similar relation has been described for the opportunistic pathogen *Streptococcus bovis*. This bacterium is thought to selectively colonize malignant and pre-malignant colonic sites by which it can cause systemic infections in susceptible individuals (see Section 5). Some contradicting results on actual *S. bovis* colonization of tumor tissue have, however, been reported. Conventional culturing techniques to determine the carriage rate of *S. bovis* in adenoma, carcinoma and healthy biopsies did not provide clear evidence for the selective colonization of adenomas or carcinomas by this bacterium (Norfleet & Mitchell, 1993; Potter *et al.*, 1998). More recently, Abdulmir and co-workers showed the presence of *Streptococcus gallolyticus* (*S. bovis* biotype I) DNA in carcinoma and adenoma tissue via polymerase chain reaction (PCR)-based techniques, which are more sensitive than conventional culturing techniques. DNA from *S. gallolyticus* was detected in about 50% of the tumor biopsies and in 35% of off-tumor tissue samples from the same patients. Strikingly, however, *S. gallolyticus* DNA was only found in <5% of the colonic tissue samples of healthy control subjects (Abdulmir *et al.*, 2010). More recently, several studies have assessed the bacterial communities in healthy, adenoma and CRC tissue by deep 16S ribosomal DNA sequencing approaches. Shen and colleagues compared the bacterial composition in normal tissue samples from adenoma patients and from individuals without colon abnormalities. The data showed increased levels of proteobacteria and decreased bacteroidetes species in off-tumor tissue samples from adenoma patients (Shen *et al.*, 2010). Interestingly, Sobhani *et al.* (2011) reported that the abundance of Bacteroides was significantly increased in tumor and normal tissue of cancer patients compared to healthy controls. More importantly, the abundance of Bacteroides was higher in tumor tissue of cancer patients than adjacent off-tumor tissue, which was paralleled by an increased IL-17/CD3 immune cell infiltration in the malignant tissues. Another recent study by Marchesi *et al.* (2011), compared differences in healthy and cancerous tissue within cancer patients and found that tumor tissue was overrepresented by species of the genera *Coriobacteridae*, *Roseburia*, *Fusobacterium* and *Faecalibacterium* that are generally regarded as gut commensals with probiotic features. On the contrary, this study found decreased colonization of *Enterobacteriaceae*, such as *Citrobacter*, *Shigella*, *Cronobacter*, and *Salmonella* in adjacent off-tumor mucosa from the same investigated patients.

The development of colorectal tumors is schematically depicted from left to right. Initiation of carcinogenesis is a process in which many factors are involved. As discussed in this Chapter, certain bacterial pathogens, bacterial toxins, or bacterial toxic metabolites (1) may contribute to the initiation and progression of CRC by causing DNA damage, induction of COX-2/IL-8, TLR signalling and/or cell proliferation pathways (2). Consequently, the altered metabolic profile of colon tumor cells and/or differential expression of bacterial receptor molecules on tumor cells (3) creates a new niche that recruits a different bacterial population (4) of which certain opportunistic pathogens can eventually breach the bowel wall and cause a systemic bacterial infection (5). The latter group of bacteria may play an important signalling function for the early detection of CRC by serological assays.

Fig. 1. Host-Microbiome interactions during CRC

5. CRC-associated bacterial infections

5.1 *Streptococcus bovis*

The most extensively studied bacterium that has a well-appreciated association with CRC concerns *Streptococcus bovis*. McCoy and Mason first reported such a case in 1951 (McCoy & Mason, 1951). In the 1970's this association was re-discovered by Hoppes and Lerner, who reported that 64% of the *S. bovis* endocarditis cases had gastrointestinal disease (Hoppes & Lerner, 1974). A few years later, Klein *et al.* (1977) reported an increased incidence of CRC in patients with *S. bovis* endocarditis. These investigators additionally discovered that fecal carriage of *S. bovis* in CRC patients was increased about 5-fold compared to healthy controls. At the time, these findings led to the recommendation to perform colonic evaluation in patients that were diagnosed with an *S. bovis* infection. Over the years, many studies have confirmed the association between *S. bovis* infection and CRC. In these studies, the prevalence of *S. bovis* infection with underlying CRC ranged from 10 - 100% (median 60%) for patients that underwent colonic evaluation (Boleij *et al.*, 2011b).

5.1.1 *Streptococcus bovis* biotypes

Based on phenotypic diversity, *S. bovis* was previously divided into three biotypes I, II.1 and II.2. Of these biotypes, biotype I is most often associated with endocarditis, while biotype II is mostly found in cases of bacteremia or liver disease. Strikingly, the association between *S. bovis* biotype I infection and CRC (21- 71%) is much higher than that of *S. bovis* biotype II (11-30%) (Corredoira *et al.*, 2008; Corredoira *et al.*, 2005; Giannitsioti *et al.*, 2007; Herrero *et al.*, 2002; Jean *et al.*, 2004; Lee *et al.*, 2003; Ruoff *et al.*, 1989; Vaska & Faoagali, 2009)(Beck *et al.*, 2008; Tripodi *et al.*, 2004). In fact, the reported incidences of carcinomas and adenomas in *S. bovis* biotype II infected patients are within the range for the normal asymptomatic population (0.3% for carcinomas / 10-25% for adenomas), whereas the rates for *S. bovis* biotype I were significantly increased (Lieberman & Smith, 1991; Lieberman *et al.*, 2000; Spier *et al.*, 2010). The distinct association of these different *S. bovis* biotypes with CRC may

have accounted for the wide range of association percentages that have been reported over the years in literature. More importantly, because most studies have not discriminated between *S. bovis* biotypes the association between *S. bovis* biotype I and CRC may have structurally been underestimated. It is important to note that Schlegel *et al.* (2003) suggested renaming *S. bovis* biotype I into *S. gallolyticus* subsp. *gallolyticus*, *S. bovis* biotype II/1 into *S. infantarius* subsp. *coli* or *S. infantarius* subsp. *infantarius* and to rename *S. bovis* biotype II/2 into *S. gallolyticus* subsp. *pasteurianus*. This new nomenclature should be used to better discriminate between the different *S. bovis* subspecies of which *S. gallolyticus* is the only species with an unambiguous association with CRC (Boleij *et al.*, 2011b).

5.1.2 *Streptococcus gallolyticus*

Recently, some striking differences between *S. bovis* biotypes were revealed that could explain their different association rates with CRC. First of all, *S. gallolyticus* seems to contain distinguished mechanisms to adherence to extracellular matrix (ECM) structures like collagen and fibrinogen (Ellmerich *et al.*, 2000a; Sillanpaa *et al.*, 2008; Sillanpaa *et al.*, 2009). Interestingly, (pre-)malignant colonic sites are characterized by displaced collagen of the lamina propria (Galbavy *et al.*, 2002; Yantiss *et al.*, 2001), through which specifically *S. gallolyticus* may colonize these sites. Besides the ECM components, also other structures at the epithelial surface may play a role in the initial adhesion to enterocytes. For example, Henry-Stanley *et al.* (2003) reported binding of *S. bovis* strains to heparan sulfate proteoglycans, which may be mediated by surface-associated HlpA (Boleij *et al.*, 2009). In an *in vitro* trans-well model containing a differentiated intestinal monolayer, the paracellular translocation efficiency of *S. gallolyticus* was shown to be significantly higher than that of other *S. bovis* biotypes. This could mean that this bacterium has an advantage over other *S. bovis* subspecies to cross an intestinal epithelium, which possibly only occurs at (pre-)malignant sites with reduced barrier function (Boleij *et al.*, 2011a). Recent data suggested that *S. gallolyticus* does not induce a strong pro-inflammatory IL-8 response in epithelial cells in contrast to other *S. bovis* strains, which may be a possibly mechanism by which *S. gallolyticus* stays rather invisible for macrophages in the lamina propria. Furthermore, Hirota *et al.* (1995) discovered that *S. gallolyticus* isolates from endocarditis patients, express human sialyl Lewis^x antigens on their cell surface unlike other fecal isolates. Mimicking human sialyl antigens, which are naturally present on monocytes and granulocytes, could therefore be a second mechanism of *S. gallolyticus* to remain unnoticed by the human innate immune system. Moreover, sialyl Lewis^x antigens could make these bacteria more efficient in binding to endothelial cells and invasion into the circulatory system (Hirota *et al.*, 1996). Finally, *S. gallolyticus* was shown to have superior efficiency to form biofilms on collagen I and IV surfaces (Boleij *et al.*, 2011a; Sillanpaa *et al.*, 2008). The latter finding could explain the increased incidence of *S. gallolyticus* as causative agent in infective endocarditis. Based on the current state-of-the-literature (July 2011), the following events in CRC-associated *S. gallolyticus* endocarditis can be envisaged **i)** *S. gallolyticus* specifically adheres to (pre-)malignant colonic sites for instance via binding to displaced collagen of the lamina propria or other tumor cell specific adherence factors; **ii)** *S. gallolyticus* may promote tumor progression by induction of the COX-2 pathway; **iii)** *S. gallolyticus* takes advantage of the distorted structure of the colonic epithelium at (pre-)malignant sites to pass the colonic wall; **iv)** *S. gallolyticus* stays relatively invisible for the innate immune system and can reach the blood stream; **v)** *S. gallolyticus* can cause a secondary infection at sites with high exposure of collagens, such as present at damaged heart valves. It should be noted, however, that many

of these data were obtained by *in vitro* studies and that it remains to be determined how this relates to the *in vivo* situation.

5.2 *Clostridium septicum*

In addition to *S. gallolyticus* endocarditis, also *Clostridium septicum* infections have been clinically associated with sporadic CRC (Chew & Lubowski, 2001; Mirza *et al.*, 2009). *C. septicum* is not considered to be part of the normal intestinal microbiota and is a rare cause of bacteremia (<1% of all cases). Hermesen *et al.* (2008) investigated 320 cases of *C. septicum* infections, 42% of which had a gastrointestinal origin. Malignant disease was present in 30-50% of these cases. The underlying mechanism of this association is not known, but it has been speculated that the hypoxic and acidic environment of the tumor specifically favor germination of *C. septicum* spores that enter the gastrointestinal tract via contaminated food (Dylewski & Luterman). A direct involvement of *C. septicum* in the development of CRC has thus far not been investigated, but it is hypothesized that *C. septicum* infections are primarily a consequence of CRC itself. Also *Clostridium perfringens* and *Clostridium butyricum* have been described in relation with CRC (Cabrera *et al.*, 1965; Rathbun, 1968). However, these strains are much less virulent than *C. septicum* and their association with CRC is less evident. Although infections with *C. septicum* are rare, underlying malignancy should be suspected and also in these cases full bowel examination could eventually save patients' lives.

5.3 *Helicobacter pylori*

Helicobacter pylori has been classified as gastric cancer-causing infective agent by the International Agency for Research on Cancer (IARC) in 1994. Most *H. pylori* strains, however, are non-invasive organism and exist in a non-adherent extracellular mucous environment. A small number of strains adheres to gastric epithelial cells, which most likely involves a number of different surface receptors (Wilkinson *et al.*, 1998). The presence of the pathogenicity island, expressing the cytotoxins VacA and CagA, is an important virulence determinant in these strains (Ekstrom *et al.*, 2001; Huang *et al.*, 2003; Crabtree *et al.*, 1994; Kuipers *et al.*, 1995). It is thought that long-term exposure to these toxins induces gastric inflammation that can eventually lead to gastric carcinomas (Higashi *et al.*, 2002; Fox, 2002). A meta-analysis conducted in 2006 by Zumkeller *et al.* indicated also a slightly increased risk for CRC (factor 1.4) in individuals with a *H. pylori* infection (Zumkeller *et al.*, 2006). Another study showed that CagA status was associated with a significantly increased risk (factor >10) for CRC among hospitalized patients that were *H. pylori* seropositive (Shmueli *et al.*, 2001). Notably, this study again underscores the importance of proper microbiological classification and characterization of cancer-associated infectious agents, since not all *Helicobacter* strains may be associated with CRC. Like has been the case for *S. bovis*, lack of proper distinction between *H. pylori* subspecies could have biased or even underestimated a possible association of this bacterium with this disease (Erdman *et al.*, 2003a,b).

6. CRC Microbiome-based Immunoassays

The occurrence of specific CRC-associated bacterial infections, as discussed in the previous section, paves the way for the development of novel diagnostic tools. In this respect, it is important to realize that *S. gallolyticus* infections occur without clinical symptoms due to its mild virulence (Haimowitz *et al.*, 2005). Clinical manifestation of *S. gallolyticus* infections in otherwise compromised patients (*e.g.* damaged heart valves), may very well only represent

the tip of the iceberg of all infections with this bacterium in individuals with (pre-)malignant colonic lesions. This notion has been the incentive to investigate whether a humoral immune response to sub-clinical *S. gallolyticus* infections could aid in the early detection of CRC. Notably, as infectious agents in general induce a more pronounced immune response compared to tumor “self” antigens, CRC-associated bacterial antigens could be instrumental in the immunodiagnosis of this disease (Tjalsma, 2011). Furthermore, several features of circulating antibodies make these attractive targets in diagnostic medicine: **i)** they reflect a molecular imprint of disease-related antigens from all around the human body, **ii)** although an antigen may be present only briefly, the corresponding antibody response is likely to be persistent, **iii)** the half-life of antibodies is about 15 days which minimizes daily fluctuations, **iv)** antibodies are highly stable compared to many other serum proteins making serum-handling protocols less stringent, **v)** the amplification cascade governed by the humoral immune system causes a surplus of circulating antibodies after appearance of the cognate (low-abundance) antigen. Several studies have shown that serum antibody levels against *S. bovis*/*S. gallolyticus* antigens could discriminate CRC cases from healthy controls (Abdulmir *et al.*, 2009; Darjee & Gibb, 1993; Tjalsma *et al.*, 2006). Interestingly, the humoral immune response to ribosomal protein (Rp) L7/L12 from *S. gallolyticus* was found to be higher in early CRC compared to late CRC stages, whereas this was not paralleled by increased antibody production to endotoxin, an intrinsic cell wall component of the majority of intestinal bacteria (Boleij *et al.*, 2010). This implies that the immune response to RpL7/L12 is not a general phenomenon induced by the loss of colonic barrier function. Furthermore, this observation could point to a temporal relationship between *S. gallolyticus* and CRC, suggesting that late stage tumors may change in such a way that bacterial survival in the tumor microenvironment is diminished. The possibility that disease progression may drive bacteria out of the cancerous tissue is similar to what has been reported for *H. pylori* during gastric cancer progression (Corfield *et al.*, 2000; Kang *et al.*, 2006). A relationship of *S. bovis* with early stages of CRC is underscored by a vast amount of case studies showing that its infection was associated with pre-malignant adenomas. These cases would have remained undiscovered if these patients did not present with an active *S. bovis* infection. Future research should be aimed at development of more specific *S. gallolyticus*-based serological assays to investigate the clinical utility of such tests for the early detection of CRC (Tjalsma *et al.*, 2006, 2008; Tjalsma, 2010). Furthermore, as CRC is a highly heterogeneous disease that is probably accompanied by even more heterogeneous microbiome shifts, accurate diagnosis based on biomarkers from a single bacterial species on the population level is highly unlikely. Therefore, future research should also be aimed at the identification of additional tumor-associated intestinal bacteria that may never have been found to cause clinical infections but do induce a humoral immune response. Furthermore, as discussed in Section 3 of this Chapter, certain mucosa-associated bacteria may be involved in CRC initiation or progression. Invasiveness of these pathogens or exposure to their antigens may elicit IgG responses that are valuable for CRC risk assessment. These individuals may not directly need bowel examination, but could be enrolled in a more strict monitoring program.

7. Conclusions

The development of CRC is a multistep process that may take over 20 years to progress from an adenoma into an advanced carcinoma. The fact that the intestinal microbiome plays an important role in this process is clearly shown by the inflammatory effects of intestinal

bacteria, which are essential to develop disease in animal models. Furthermore, accumulating evidence suggests that bacterial production of toxins, toxic metabolites and the direct influences on pro-carcinogenic pathways in host epithelial cells are contributing factors that promote the accumulation of mutations that may eventually lead to carcinomas. However, still many questions remain to be answered. For example, our knowledge on the impact of CRC on the local intestinal microbiota and *vice versa*, is still in its infancy. Future research should focus on the detailed mapping of the microbiota in close proximity of early adenomas and carcinomas. These local changes in microbiota may for instance provide clues in the understanding why only 10% of the adenomas progress into carcinomas. Such knowledge could give us new leads for cancer diagnosis, for example by using signaling bacteria, such as *S. gallolyticus* that benefit from the altered tumor environment, as diagnostic targets. Furthermore, this knowledge could provide leads for the selective removal of high-risk bacterial populations by health promoting species, as a new strategy in CRC prevention. Altogether, this Chapter points out that the colonic microbiota should be regarded as an important factor in intestinal carcinogenesis. Further research in this field is crucial to fully understand the etiology of CRC and has a high potential to lead to new diagnostic tools and therapeutic interventions.

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Colorectal cancer is a common disease, affecting millions worldwide and represents a global health problem. Effective therapeutic solutions and control measures for the disease will come from the collective research efforts of clinicians and scientists worldwide. This book presents the current status of the strides being made to understand the fundamental scientific basis of colorectal cancer. It provides contributions from scientists, clinicians and investigators from 20 different countries. The four sections of this volume examine the evidence and data in relation to genes and various polymorphisms, tumor microenvironment and infections associated with colorectal cancer. An increasingly better appreciation of the complex inter-connected basic biology of colorectal cancer will translate into effective measures for management and treatment of the disease. Research scientists and investigators as well as clinicians searching for a good understanding of the disease will find this book useful.

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