Chapter 8

Can the oral microflora affect oral ulcerative mucositis?

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Introduction

Chemotherapy and radiotherapy are of the first choice treatment option for several common malignancies because of their tumor damaging effects. These therapies, however, produce a wide range of detrimental toxicities. One of the best studied toxicities is oral mucositis. Hematopoietic stem cell transplant (HSCT) patients undergo chemotherapies with or without radiotherapy just before the transplantation. Between 70 and 89% of the HSCT patients experience oral mucositis (1, 2) and virtually all patients that undergo radiotherapy for treatment of head and neck cancers (3). Oral ulcerative mucositis, the most severe form of mucositis, is associated with significant pain, leading to an increased use of analgesics, more tube feeding, more febrile days, and a longer stay in the hospital (4). Mucositis can have such negative effect on the quality of life of patients that main treatment needs to be delayed, which can lead to poorer treatment outcomes (5).

The sequence of oral mucositis consists of five phases: initiation, the primary damage response, signaling and amplification, ulceration, and healing (6). These five phases are not distinct, they overlap in time, and several phases may occur at the same time. The cause of mucositis results from a series of complex biological events combined with the influence of the host including the microbial environment. Sex, age, weight, comorbidities, and lifestyle are host factors that are traditionally linked to mucositis risk. Today, studies on the genetic factors and the immune response as risk factors are gaining more attention. Another important host factor is the microflora. The oral microflora is thought to be a modulating factor for mucositis. The microflora colonizes the ulcerative lesions and aggravates their severity (7). Yet, the exact relationship between specific bacterial species and ulcerative mucositis remains unclear (8).

Ulcerations may be more painful, more extended, may last longer, or may heal worse when the oral microflora is out of balance and harmful bacteria are overgrowing the oral biofilms. This review will give an overview of the recent literature that focuses on the role of the microflora influencing mucositis. Is there currently evidence that the microflora is able to influence mucositis, and more specifically, are bacteria able to influence the healing of wounds and ulcerations in the oral cavity?

The oral microbiome

The microbial composition of the oral cavity is highly complex. In recent
years, the focus changed from studying specific pathogenic bacteria to a more environmental and holistic approach (9). Because of new methods, such as next-generation sequencing (NGS), we know that traditional culturing techniques cannot detect all microbial species in oral biofilms. It is suggested that in the oral cavity, at least 50% of the species are uncultivated and uncharacterized (10). There is yet lack of knowledge about the function of the uncultivated microorganisms in the oral ecosystem and whether they are good or bad microorganisms. In 2005, Rasiah et al. (11) showed, by using a DNA-based DGGE detection technique, that the microbial composition of 10 healthy individuals did not change over the period of 7 years. These results have been confirmed by next generation sequencing techniques, although for a shorter period of time (12). It is, therefore, accepted that the composition of the oral microbiome differs strongly between individuals, but is extremely stable in health within one subject (11-13), suggesting that (oral) health is a stable ecological situation and this stability may be lost in disease.

From recent ecological studies, we know that the association of some oral bacteria with diseases such as caries or periodontitis is less clear than previously thought and the term ‘good’ and ‘bad’ microorganisms is more or less abolished. For example, periodontitis was thought to be associated with a relative small group of bacteria; however, studies using NGS techniques on 16S RNA, which provides operational taxonomic units of an ecosystem, indicate that its cause is far more complex (14). Moreover, we are now at the beginning of a new era where ecological functions can be assigned to new found, not culturable microorganisms based on their transcribed RNA using metagenomic (shotgun) sequencing (15). Such a study in periodontitis patients revealed that several of the old group of pathogens, such as Prevotella and Porphyromonas species, are indeed associated with the disease; however, many more species seem to be involved. Moreover, the presence of certain proteolytic pathways and the related genes make a periodontitis ecosystem more prone to tissue inflammation (16). Interesting in this study is that in microbiomes from periodontic patients there is a significantly overrepresentation of genes that are associated with inflammation processes, such as bacterial chemotaxis, biosynthesis of lipopolysaccharides, flagellar assembly, and collagenase activity. Moreover, genes associated with membrane transport and metabolism were underrepresented in periodontal active microbiomes. The results from this study confirm the essential role of the human inflammatory system in periodontal patients. When combining taxonomic and functional sequencing data, it was found that certain bacteria cluster in specific pathways. This finding suggests that there still is a correlation between specific bacteria, their function in
maintaining the ecosystem, and health or disease, which suggests that the ‘old microbiological knowledge’ still has its value (17, 18).

The intestinal tissue in response to the microflora

The relationship between epithelial integrity, general health, and the microflora is more extensively studied in the intestine than in the oral cavity. Like in the oral cavity, there is a constant contact of the microflora with the epithelial cell layer of the intestine. The bacteria recognizing Toll-like receptors (TLRs) and nod-like receptors on the epithelial cells are constantly challenged by the commensal flora. This implies that normally, the human intestine is in a state of low-grade inflammation. This chronic inflammation is a normal reaction of the host to protect itself from infection. It is responsible for both pathogen killing and tissue repair processes (19). There is evidence that misbalance in the intestinal microflora is associated with inflammatory diseases such as inflammatory bowel disease, pouchitis, radiotherapy-induced diarrhea, atopic disease, obesity, and diabetes (20, 21).

In contrast to the above-named inflammatory processes due to the microflora, there is also evidence that the commensal intestinal flora has a protective function against inflammation (21, 22). The bacteria *Bacteroides thetaiotaomicron*, *Bifidobacterium infantis*, *Lactobacillus salivarius*, and *Faecalibacterium prausnitzii* have the capacity to lower the inflammatory reaction in epithelial cells (23-25). Lactobacilli play an important role in maintaining or restoring the intestinal epithelial integrity. They stimulate the upregulation of epithelial tight junction proteins that restore the intestinal barrier function (26). The intestinal mucus layer protects the underlying tissues against infections and inflammation, and *Lactobacillus* species are able to increase the expression of MUC-2 and MUC-3 genes that enhance the mucus layer (27, 28). Furthermore, *Lactobacillus rhamnosus* GG (ATCC 53103; LGG) has the capacity to inhibit cytokine-induced apoptosis and promote cell growth (29). Especially the protein, LGG-p40, which is also found in other *Lactobacillus* spp., is able to activate the epidermal growth factor receptor (EGFR), which on its turn will activate an intracellular target called Akt. Intestinal injury and acute colitis are the result of epithelial apoptosis and disruption of the barrier function in the gut. Activation of Akt will inhibit the apoptosis and restores the barrier function, which on its turn has a positive effect on the epithelial tissues in several colitis models (29, 30). This and earlier evidence of the positive effect of the use of Lactobacilli led to the new Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO) guideline that suggests the use of probiotic *Lactobacillus* spp. to prevent chemotherapy- and
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Radiotherapy-induced diarrhea patients with malignancies in the pelvic region (31). Finally, a number of intestinal bacteria produce butyrate that has profound anti-inflammatory effects in the intestine. Butyrate stimulates the migration of epithelial cells from the gut and thereby wound healing. In contrast, butyrate is suggested as a virulence factor in the oral cavity. It harms periodontal ligament cells and fibroblasts by the induction of toxic reactive oxygen species (16, 32-34). Chemotherapy and antibiotic treatment greatly influence the composition of the intestinal microflora (21, 35, 36). The total number of fecal bacteria decreases (37, 38), some opportunistic pathogens such as *E. coli* and *Enterobacteriaceae* grow out, whereas other beneficial bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. decrease in numbers (39, 40). Recently, two authors argued that the intestinal microflora may even have a direct influence on the pathophysiology of intestinal mucositis (21, 35). Because of the conflicting data on the protective effect of butyrate and the fact that from these bacteria, only *L. salivarius* is an inhabitant of the oral cavity, it is unlikely that these bacteria do play a similar protective role for the oral mucosa.

The oral flora and periodontal disease

The oral cavity harbors several habitats that differ ecologically, depending on the location and substratum. There are many factors that determine which microorganisms can survive and establish in a local habitat (41). An important substratum in the oral cavity is the epithelial cell surface, where, similar to the intestine, the microorganisms are in close contact to and stimulate the immune system. The ecological interaction between the microorganisms and the host determines whether the host will respond and is able to maintain homeostasis. Under certain circumstances, specific microorganisms can grow out and disturb the homeostasis, which can lead to diseases such as periodontitis. For periodontitis, this mechanism has been recently reviewed (42).

Periodontitis is a chronic inflammatory disease of the tooth-supporting tissues that leads to loss of connective tissue and bone support and ultimately leads to tooth loss (43). In periodontitis, the balance between microbial and inflammatory processes in subgingival sites gets disturbed by the accumulation of large amounts of plaque. Gingival crevicular fluid flow increases, which provides nutrients for proteolytic bacteria like *P. gingivalis*. As a consequence of proteolytic activity, the local pH will increase and the redox potential falls, which promotes the growth of more anaerobic and proteolytic bacteria (41). The proteolytic activity in the pocket leads to cleavage of host defense molecules. This results in a raised host immune response that is then followed by additional damage to the tissues. This process is characterized by a shift in
the subgingival microflora that is accompanied by an increase in species richness and diversity. The numbers of Gram-negative and anaerobic bacteria increase drastically. Studies based on DNA sequencing of the plaque reveal that apart from known periodontal pathogens such as *P. gingivalis* and *Actinobacillus actinomycetemcomitans*, a fair portion of the bacteria in diseased sites is yet unculturable (41, 44). In a healthy oral habitat, the periodontal pathogens have a noncompetitive relationship to other commensal oral species (44).

The bacterium *P. gingivalis* is associated with the chronic form of periodontitis as it is found in diseased sites and only present in low numbers in periodontally healthy individuals (45, 46). It is a Gram-negative, anaerobic, black-pigmented, non-motile, asaccharolytic, rod-shaped bacterium. *P. gingivalis* has the ability to invade oral tissues where it can replicate intracellularly. In this way, *P. gingivalis* escapes the immune system and is successfully supported in the host tissues (47, 48). Moreover, *P. gingivalis* is positively associated with oral ulcerative mucositis, which makes this bacterium interesting to study in more detail (2).

**Virulence factors of *P. gingivalis* and the host response**

*P. gingivalis* possesses a number of well described virulence factors. As it is a Gram-negative bacterium, its cell wall contains lipopolysaccharide (LPS). LPS activates cellular receptors from the TLR family of the host and generally triggers an immune response. LPS from *P. gingivalis* stimulates the production of a wide range of proinflammatory cytokines (49-51). Furthermore, LPS increases the level of bone resorption (52, 53). LPS from different bacterial species can vary strongly in its structure and LPS from *P. gingivalis* elicits a weaker immune response compared with other Gram-negative bacteria such as *E. coli* (54). Additionally, some forms of LPS from *P. gingivalis* may act as an agonist, whereas other forms of LPS act as an antagonist of TLR4 (55).

Most *P. gingivalis* cells are surrounded by an antigenic capsular polysaccharide (CPS), whereas some strains do not produce CPS (56). Different *P. gingivalis* CPS serotypes elicited different IgG immune responses (57). Furthermore, encapsulated strains were more resistant to phagocytosis than non-encapsulated strains (58). Moreover, the absence of the CPS leads to a higher proinflammatory response (59) and resulted in better adherence to epithelial cells than encapsulated strains (60). Although different serotypes of CPS are involved in the regulation of the host response, the exact mechanism remains to be elucidated (45).

The cell wall of *P. gingivalis* contains long protein structures that stick out of the cell wall, called fimbriae. The long fimbriae enable the bacterium to
adhere to, among others, other bacteria and salivary components and to invade host tissues (61). The short fimbriae mainly induce cytokine expression (62). Long and short fimbriae are able to induce the production of the proinflammatory cytokines IL-1, IL-6, IL-8, and TNFα and the matrix metalloproteinase MMP-9 (63-65).

Furthermore, *P. gingivalis* possesses gingipains, a group of cysteine proteinases that account for 85% of the proteolytic activity of the bacterium (66). Gingipains have stimulating and inhibiting effects on the immune system. They may be involved in the binding of *P. gingivalis* to host cells and they may affect the vascular permeability and bleeding at periodontal sites (45). Similar as the effect of LPS from *P. gingivalis*, the effect of the gingipains on the immune system seems multiple. However, the contradictory effect may be explained by a concentration gradient of gingipains in the tissue (53).

Overall *P. gingivalis* developed several strategies to invade the host and to persist in a specific niche (45). The stimulating and inhibiting effects on the immune system may result in bypassing the host defense mechanisms so it is able to survive better in the host tissues (67).

**Role of the oral flora in oral mucositis**

Studies into the effect of the oral microbial environment on mucositis are usually of descriptive nature. Qualitative and quantitative changes in several bacterial species following cancer treatment are described and linked to oral mucositis. Recently two articles were published on this topic (2, 68). Laheij et al. (2) included 49 adult HSCT patients in their prospective study. Twice weekly, oral mucositis was scored clinically and at the same time oral rinsing samples were taken. Patients were followed from HSCT until discharge from the hospital. The rinsing samples were screened using for bacteria that are classically associated with periodontitis using real-time PCR. The bacteria in the oral cavity were statistically linked to ulcerative mucositis using longitudinal data analysis (GEE technique). In particular, *P. gingivalis* and also *P. micra*, *T. denticola*, and *F. nucleatum* were positively associated with ulcerative mucositis. The risk of having ulcerative mucositis was increased when these bacteria were present in the oral cavity. This relationship can be one of the explanations of a possible association between mucositis and periodontitis (69, 70).

Ye et al. (68) included 37 pediatric patients who received chemotherapy for several malignancies. Mucositis was scored clinically and two oral mucosal samples were taken, before and during chemotherapy. Mucosal samples were analyzed using 16S-based 454 pyrosequencing. Patients who later developed mucositis had a higher microbial diversity before chemotherapy, and there was
higher intersubject variability compared with patients who did not develop mucositis. Furthermore, the change in bacterial composition was more pronounced in patients who later developed mucositis.

However, from literature, there is no clear association between specific bacterial species and mucositis. Differences between studies existed in bacterial strains studied, study population, collection time, sampling methods, and scoring methods for mucositis. Therefore, it is not possible to draw detailed conclusions from all of these studies (8).

Another way to investigate the role of the oral microflora is to study the influence of several antimicrobial agents on mucositis. The hypothesis is that reducing (part of) the oral flora reduces mucositis. The antimicrobial mouthwash chlorhexidine and several systemic and local antimicrobials have been screened, and the efficacy of these interventions in preventing mucositis after cancer treatment did not show evidence in favor of any of the products (71, 72). However, this does not mean that the oral flora plays an insignificant role in modulating mucositis. Specific oral bacteria may have more subtle pathways to influence mucositis. Some pathways may be similar to pathways in the gut, whereas others may be specific for the oral cavity. An interesting pathway may be the influence of bacteria on the healing of ulcerative lesions in the oral cavity. Bacterial cells outnumber human cells in the body and humans have a synergistic relationship with their microflora. It is the balance between microorganisms and the host that keeps the human body healthy. Simply eliminating part of the microflora with antimicrobial agents results in a disbalance in this ecosystem and, in the case of ulcerative mucositis, bacteria such as P.gingivalis can continue their virulent processes.

**Oral bacteria and wound healing**

In the oral cavity, the use of chemotherapy, radiotherapy, and antibiotics changes the microbial composition during HSCT treatment (8, 68). In a recent study, using a real time PCR method, several bacteria, and mainly P. gingivalis were found to be associated with oral ulcerations after HSCT (2). Details on this study are described in the previous paragraph. Bacteria may have an effect on the healing of the ulcerations once the lesions are established. Bacterial butyrate stimulates the migration of epithelial cells and wound healing (34) and Lactobacillus rhamnosus GG promotes cell growth by reduction of the epithelial apoptosis due to one of its excreted proteins in the gut, but these bacteria can also be found in the oral cavity (29).

The healing of wounds occurs in three distinct and overlapping phases in time
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and location: inflammation, cell proliferation, and remodeling of the tissues (see Fig. 1). The inflammatory phase starts with the attraction of neutrophils, macrophages, and lymphocytes (73). In the literature, there is debate about the necessity of the immune response for effective wound repair. Oral mucosal wounds heal faster and with less scar formation than dermal wounds. Wounds in the oral mucosa have low numbers of nearly all immune cells (74). It suggests, therefore, that a lower immune response results in better healing of wounds (74, 75). As is stated earlier, the bacterium *P. gingivalis* is able to raise the host immune response but it can also dampen the immune response, thereby enabling itself to survive in host tissues (45). So it is possible that *P. gingivalis* influences the healing of wounds via the immune system.

**Figure 1** Mechanism of wound healing. This is the final stage of oral mucositis in which the inflammatory response is downregulated (H) and epithelial cells start to migrate and proliferate (G) to close the ulcerative wound. Submucosal cells (A) will regenerate and this process will produce new tissue, which is not exactly similar to the old tissue (scar tissue). A is the dermis, B the epidermis, C the fibrin clot, D the wound, E the granulation tissue, and F the blood vessels. Adapted from Singer [73] and Sonis [7]

Re-epithelialization of the wound starts within hours after the formation of the wound. Epithelial cells migrate over the viable tissue, separating it from the nonviable tissue. After 1 or 2 days, the epithelial cells start to proliferate underneath the migrating cells. The stimuli that determine the migration and proliferation of epithelial cells are not yet understood. After about 5 days, granulation tissues starts to form in the underlying tissues, and finally, the tissues are remodeled, leaving scarred tissue (73).
Recently, we showed that several oral bacteria are capable of inhibiting the migration of oral epithelial cells \textit{in vitro} (76). Closure of a scratch made in the oral epithelial cells was about 30 – 25% in the presence of a high concentration of heat inactivated \textit{P. gingivalis} and \textit{Prevotella nigrescens} compared with 100% for control. Closure of the scratch in the presence of heat inactivated \textit{Streptococcus mitis}, \textit{Tannerella forsythia}, and \textit{P. intermedia} varied from 51 – 72%.

Moreover, a high concentration of viable \textit{P. gingivalis} cells and growth medium that contained secreted molecules from \textit{P. gingivalis} even inhibited cell migration totally. Others reported the inhibiting effect of \textit{P. gingivalis} on epithelial cell migration as well (77-79). As epithelial cell migration is an important step in wound healing, oral bacteria in general and \textit{P. gingivalis} more specifically may influence the healing of oral ulcerations via this route as well.

\textbf{Conclusion}

In recent years, the idea that health and disease conditions are associated with a shift in the microbial composition of the biofilm that colonizes the tissue emerged. Under chemotherapy or radiotherapy, opportunistic bacteria can grow out, leading to or aggravating unpleasant toxicities such as mucositis. The intestinal microflora seems to influence mucositis via several pathways. In the oral cavity, bacterial species that are associated with periodontitis seem to be able to delay the healing of wounds and, therefore, influence mucositis. Therefore, the association between microorganisms and mucositis seems more subtle and complicated than was thought before.

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