Review Article

Gram-positive microorganisms in periodontitis

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ABSTRACT

The microbial etiology of periodontal disease has been the focus of researchers for a long time. The hunt for the pathogens of the periodontal diseases is underway for more than 100 years and endless up today. Despite the increasing knowledge about oral microbiota, we are not able to implicate any one particular organism that can be considered as a candidate pathogen. In fact, the term "candidate pathogen" has lost its steam with a myriad of microorganisms being incriminated from time to time. Many studies, the bacterial etiology of periodontitis has used either culture-based or targeted deoxyribonucleic acid approaches and so it is likely that pathogens remain unidentified. The arrival of 16S cloning and sequencing has facilitated identification of several uncultivable bacteria in the oral cavity. Periodontal diseases have been traditionally associated with anaerobic Gram-negative microbiota. However, improvements in sampling handling and culture media have revealed that Gram-positive anaerobes belonging to the genus *Eubacterium* can make up around half of microbiota in advance disease. The present review highlights some Gram-positive microorganisms known bacteria associated with periodontal destruction.

KEY WORDS: 16S cloning, Periodontal diseases, Real-time polymerase chain reaction

INTRODUCTION

It is common that destructive periodontal diseases are infections caused due to bacteria that colonize the tooth surface, gingival margin, and subgingival environment. Many multiple studies in the past decades accompany the unique infectious nature of chronic periodontitis and aggressive periodontal diseases. It has been demonstrated, however, that the initiation and progression of the inflammatory and destructive periodontal lesion is related not only to the presence of bacterial strains pathogenic for the periodontium but also to the lack or minimal proportions of the beneficial microorganisms in a susceptible host.[1-4] Periodontal diseases are polymicrobial, multifactorial diseases, and there are many host factors involved in determining the individual susceptibility to disease. It is recognized that the relationship between periodontal microbiota and the host is generally benign, but when the specific bacterial species overgrows in the subgingival spaces, this may cause periodontal inflammation and destruction with attachment loss and bone loss.

Periodontal diseases are inflammatory diseases of the periodontium caused by bacterial accumulation in oral biofilms and are broadly classified by the severity and symptoms of the disease into gingivitis and periodontitis. Gingivitis, like dental caries, is among the most common chronic infections of humans worldwide. The etiology of gingivitis has been extensively detailed, with a lack of proper oral hygiene being a primary factor in the occurrence of the disease. In state of health, the gingival sulcus is primarily colonized by the Gram-positive microorganisms, which occupy various *Streptococcus* and facultative *Actinomyces* species. However, in the absence of proper oral hygiene, there is a dramatic increase in the proportion of Gram-negative bacteria, which activate the host inflammatory response through lipopolysaccharide and other destructive enzymes. The clinical signs of gingivitis include gingival inflammation, bleeding on probing, and spontaneous bleeding.[5-7]

The uncertain taxonomy of oral anaerobic Gram-positive bacilli and their generally slow-growing nature has limited the understanding of their role in periodontal disease. The current objective was to design and use species-specific oligonucleotide probes to investigate the relationship of selected Gram-positive anaerobic bacilli to periodontal disease.[8] The study of the microbiota associated with periodontal diseases has also been impacted by changes in paradigms

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regarding the etiology and pathogenesis of periodontal diseases over the years. In recent years, much attention has been given to the essential role of inflammation and other immune mechanisms in periodontal disease pathogenesis. These studies are fundamental to our understanding of the complex mechanisms involved in these multifactorial diseases.[9] In 1994, Socransky and Haffajee argued that periodontal disease initiation and progression required the simultaneous occurrence of a number of factors: (1) The virulent periodontal pathogen, (2) the local environment, and (3) host susceptibility. Later, Page and Kornman expanded this model to acknowledge the contributions of genetic and acquired risk factors.

Recently, contrary to the "dogma" of Gram-negative bacterial dominance in periodontitis, Gram-positive anaerobic species exhibit a significant increase in deep diseased sites relative to healthy sites and can be detected in greater abundance than Gram-negative species in some studies. Many of these species are as yet to be cultivated. These organisms exhibit polymicrobial synergy. Newly identified disease-associated species include the Gram-positive Filifactor alocis and *Peptostreptococcus stomatis* and other species from the genera *Prevotella, Megasphaera, Selenomonas, Desulfobulbus, Dialister,* and *Synergistetes*.[10]

Gram-positive bacteria are bacteria that give a positive result in the Gram stain test, which is traditionally used to quickly classify bacteria into two broad categories according to their cell wall. Gram-positive bacteria absorb the crystal violet stain used in the test and appear to be purple colored when seen by microscope. This is because the thick peptidoglycan layer in the bacterial cell wall retains the stain after it is washed away from the rest of the sample, in the decolorization stage of the test. They can be broadly classified into cocci and rods. They are aerobic or anaerobic.[11] Gram-positive anaerobic cocci have been isolated from a wide range of human infections constituting one-fourth of anaerobic species from clinical specimens. Most infections involving Gram-positive anaerobes are polymicrobial and appear to involve synergistic interactions with other bacteria. Previously epidemiologic evidence linked *Peptostreptococcus* spp. with dental infections but now its association with periodontal diseases is also becoming evident.[1,10]

Several studies had shown the significance of Gram-positive anaerobes such as *Peptostreptococcus* spp., *Parvimonas micra* (previously *Peptostreptococcus* micros), *Eubacterium nodatum,* Filifactor alocis, and *Streptococcus sanguinis* in periodontal disease.

In a study by Kumar *et al.* detected a large number of uncultivated *Peptostreptococci* which is Gram-positive anaerobic cocci. The association of *Peptostreptococci* with periodontitis was robust and was far more numerous than the Gram-negative bacteria associated with periodontitis. The Gram-positive rod, Filifactor alocis, was also significantly elevated in disease subjects.[10]

In another study by Daniluk *et al.* found that, in adult patients with periodontal disease, Gram-positive anaerobes, including *Peptostreptococcus*, were the predominant bacteria in the subgingival plaque. While in the supragingival plaque, Gram-positive aerobic cocci (*Streptococcus* and *Staphylococcus*) were predominant.[12]

The study by Booth *et al.* stated that *E. nodatum* and *S. exigua* were associated with clinical indicators of periodontal disease.[8]

MOLECULAR METHODS

Microbiological culture method is one of the most broadly used tools for microbiological identification and is considered a "gold standard" due to its ability to detect new bacterial species and to test their susceptibility to antibiotic agents. However, this method has some drawbacks. It has very low sensitivity, from 103 to 104 vital bacteria are necessary to form a culture and it is especially difficult to cultivate anaerobic species. The microorganisms related to the periodontal disease, in turn, are predominantly anaerobic and are very difficult to cultivate.

In recent years, several culture-independent techniques of molecular microbial analyses have been considered. They are mainly (i) polymerase chain reaction (PCR)-based methods including single-target PCR, multiplex PCR, and quantitative PCR, (ii) DNA-DNA hybridization methods such as *in situ* hybridization, checkerboard hybridization, and 16S ribosomal RNA-based microarrays, and (iii) sequencing methods including the latest, next-generation sequencing techniques such as pyrosequencing, real-time single-molecule DNA sequencing, and nanopore-based sequencing.[13] The analysis of the contemporary microbiological diagnostic methods suggests the PCR approach as the most appropriate approach for the identification of specific pathogens due to its better sensitivity, whereas the *in situ* hybridization is applied more in research to solve certain scientific tasks.

REAL-TIME PCR

Real-time PCR, also referred to as quantitative PCR, quantitative reverse transcription-PCR, reverse transcription-quantitative PCR, and kinetic PCR, is a method used to quantify the copy numbers of DNA in clinical samples. There are two types of real-time PCR, namely an intercalator-based method and a probe-

based method. The intercalator-based method, also known as the SYBR Green method, intercalates SYBR Green, which binds to newly synthesized double-stranded DNA producing a fluorescently labeled PCR amplicon. The probe-based method, or TaqMan PCR, is more specific in that it utilizes a fluorogenic-labeled probe that binds only to its complementary sequence in the internal portion of the generated PCR amplicon. Real-time PCR has been used to detect and quantify several periodontal pathogens including *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, the tetQ gene, and total bacteria, in clinical samples.[14]

The main advantage of real-time PCR over PCR is that real-time PCR allows to determine the initial number of copies of template DNA (the amplification target sequence) with accuracy and high sensitivity. Real-time PCR results can either be qualitative presence and absence of order quantitative (copy number). In addition, real-time qPCR data can be evaluated without gel electrophoresis, resulting in reduced bench time and increased throughput. Finally, because real-time qPCR reactions are run and data are evaluated in a unified, closed-tube qPCR system, opportunities for contamination are reduced and the need for post-amplification manipulation is eliminated in qPCR analysis.

DISCUSSION

Characteristics of a Few Gram-positive Anaerobes Commonly Identified in Periodontal Disease and Evidence Linking them to Periodontal Disease

E. nodatum is an obligate anaerobe, filamentous, or club-shaped asaccharolytic, Gram-positive rod. They grow steadily in culture, biochemical, or morphological characteristics with other well-known species of the anaerobic bacteria. They detailed virulence factors such as esterases, acid phosphatases, and aminopeptidases and had been isolated from the significant proportion of subgingival microbiota of chronic periodontitis ranging from 10.8% to 54%.[15] Three species, *E. nodatum, Eubacterium timidum*, and *Eubacterium brachy*, have been described, primarily from subgingival samples taken from patients with moderate and severe periodontitis.[14]

P. micra species of the orange microbial complex set forward by Socransky *et al*.[15] The presence of *P. micra* had been positively associated with periodontitis over the past two decades. Previously known as *P. micros*, this Gram-positive, microaerophilic coccus is usually associated with polymicrobial infections such as intracranial abscesses, sinus infections, and periodontitis. *Peptostreptococcus* sp. is colonizers of the oral cavity, vagina, skin, gastrointestinal tract, (GIT) and urinary tract. These are also found that they cause systemic infections such as abscesses and necrotizing tissue infections and other infections of GIT and urinary tract in immune-compromised patients. *P. micra* possesses several virulence factors that subscribe to its pathogenic potential. The cell wall of *P. micra* had been shown that they induce a potent inflammatory reaction in macrophages.[16] They elaborate proteases that permit it to penetrate to the basement membrane.[17] It also makes a carbohydrate-mediated coaggregation with *Fusobacterium nucleatum* and *P. gingivalis*.[16] These data suggest that *Peptostreptococci* may play a role in preventing wound healing in chronic disease and may be important in the physical structure of a disease-associated biofilm.

Filifactor alocis is a meticulous, Gram-positive, obligatory anaerobic rod owed trypsin-like enzymatic activity similar such as *P. gingivalis* and *Treponema denticola*. It also has the ability to survive in periodontal pocket and shares same virulence properties with the *Fusobacterium*. Filifactor alocis (ATCC 35896T) was first isolated in 1985 from the human gingival crevice as *Fusobacterium alocis* and later reclassified as a Filifactor alocis. Oxidative stress resistance sialidase activity exhibited by *F. alocis* upshot in release of sialic acids that scavenge oxidative stress in the periodontal pocket.[18] The fastidious nature of this organism has contributed to its low detection in culture-based methods. The organism is associated to cause endodontic infection and periodontal destruction. This organism has been found in elevated numbers in aggressive periodontitis (77.8%) and chronic periodontitis (76.7%) compared with periodontally healthy individuals due to its potential to withstand oxidative stress and inflammatory microenvironment provided by periodontal pocket.[15]

A study by Dahlén and Leonhardt[15] concluded that *F. alocis* should be connected to the 12 species used for regular diagnostics of periodontitis-associated bacterial flora. This is one of the marker organisms and is considered as a salient periodontal pathogen. The organism is now identified to be significant to the pathogenic structure of biofilms associated with periodontal inflammation.[17,19] In collation with the other traditional periodontal pathogens, the elevated incidence of *F. alocis* in the periodontal pocket compared with its absence in healthy individuals or those who are periodontitis resistant have highlighted its importance in the infectious disease process.[15,20]

S. sanguinis is Gram-positive cocci they are non-motile and non-spore-forming pili of *S. sanguinis* bind to salivary α-amylase and contribute to the formation of biofilm on saliva-coated surfaces. They commence aggregation of other oral bacteria and maturation of dental plaque. Sortase A of *S. sanguinis* has an

influence on the expression of various cell surface virulence factors.

Streptococcus parasanguinis is Gram-positive, non-motile, and non-spore-forming cocci. They are facultative anaerobes. The long peritrichous fimbriae of *S. parasanguinis* are critical for the formation of biofilms on solid surfaces.[21] It is one of the prominent early colonizers of dental surfaces in the human oral cavity. Fim A protein is a potential virulence factor.

E. nodatum is branched, filamentous, Gram-positive bacteria that are non-motile and do not produce spores. When colonized, the rod-shaped bacteria bunch together in the broth cultures. When they form these clumps under favorable conditions, a biofilm is created. After incubation, cells form circular and raspberry-shaped colonies that are cream colored. *E. nodatum* is obligately anaerobic, non-saccharolytic, grow optimally at 37°C and can produce butyrate and acetate. *E. nodatum* can also hydrolyze arginine and lysine and thus use these as substrates for energy sources.

Eubacterium species have only recently been described; little work has been done in investigating these organisms for possible virulence factors. Preliminary work (unpublished data) suggests that the asaccharolytic species do not produce proteases but exhibit acid phosphatases, esterases, and aminopeptidases. *Eubacterium* C2 has been established in continuous culture where it has been shown to produce a wide range of aminopeptidase activity. Since these organisms are always found in mixed infections, they may act in concert with other bacteria in the degradation of host tissues.[15]

Oral biofilms produce various oral infectious diseases like periodontitis and others like dental caries. Asaccharolytic anaerobic Gram-positive rods (AAGPRs) (Role of AAGPRs on periodontitis) and periodontopathic bacteria, such as *P. gingivalis*, are frequently components of subgingival biofilms. However, AAGPRs are not easy to study because they are difficult to culture and produce few metabolic products; thus, the contributions of AAGPRs to periodontitis have yet to be determined. Herein, we describe the growth and formation of AAGPR biofilms and the effect of AAGPRs on the induction of cytokines from human gingival fibroblasts stimulated with periodontopathic bacteria. We also discuss the role of AAGPRs in the progression of periodontitis. Finally, we suggest future avenues of research that will be vital for improving our understanding of the mechanisms by which periodontal disease progresses.[22]

Role of AAGPR in Periodontal Diseases

AAGPRs indicate that they play an important role in promoting the formation, maturation, prolongation, and stabilization of oral biofilms, despite the fact that they do not form biofilms by themselves. In addition, in accumulated biofilms, AAGPRs do not appear to stimulate host cell immune activity and, in fact, inhibit the production of cytokines by HGF stimulated by other bacterial species. Accordingly, it is possible that AAGPR species may prolong inflammation and cause chronic periodontal disease. Further work is necessary to study the potential pathogenic mechanisms of AAGPR species, in particular, the mechanism by which they prolong the lifespan of *P. gingivalis* biofilms and accelerate the formation of *F. nucleatum* biofilms. Furthermore, it is important to document the factors responsible for promoting AAGPR growth in *P. gingivalis* culture. More details are needed on the relationship between AAGPRs and host cell immune responses. The nature of the relationships between AAGPR species and periodontopathic bacterial species to understand the mechanisms by which periodontal disease progresses.[22]

CONCLUSION

As visible from the above literature review, a number of Gram-positive anaerobes such as Filifactor alocis, *P. micra*, *E. nodatum,* and *S. parasanguinis* are now regard as potential periodontal pathogens. However, these findings are not consistent across all studies. The feasible contributory factors to this variability are study design, the population studied and the procedures of recognition of microorganisms. Furthermore, we need to keep in mind while interpreting the results of association studies, the "causal versus casual" concept. Analysis of the associations based on the Hill's criteria of causality can give us an insight into this aspect. The paradigm shift in the understanding of periodontal pathogenesis is attributed to the introduction of novel theories about the ecological events associated with periodontal destruction. The polymicrobial synergy and dysbiosis model makes us query the individual role of these Gram-positive anaerobes implicated in periodontal pathogenesis.[15,18,23,24] This has to be confirmed by future studies. Whether they fulfill all of Koch's postulates in being an infectious organism is yet to be studied.

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