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Cardiovascular disease and the role of oral bacteria

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In terms of the pathogenesis of cardiovascular disease (CVD) the focus has traditionally been on dyslipidemia. Over the decades our understanding of the pathogenesis of CVD has increased, and infections, including those caused by oral bacteria, are more likely involved in CVD progression than previously thought. While many studies have now shown an association between periodontal disease and CVD, the mechanisms underpinning this relationship remain unclear. This review gives a brief overview of the host-bacterial interactions in periodontal disease and virulence factors of oral bacteria before discussing the proposed mechanisms by which oral bacterial may facilitate the progression of CVD.

Keywords: *oral bacteria*; *cardiovascular disease*; *systemic inflammation*; *cross-reactivity*; *heat shock proteins*

Published: 21 December 2010

Cardiovascular disease (CVD) refers to diseases of the circulatory system, including outcomes such as myocardial infarction and stroke (1). CVD, along with cancer, is a leading cause of death in Western societies. According to Queensland Health, CVD was the second highest cause of disease burden in Queensland in 2003, accounting for 17.8% of the overall burden of disease (number of lives lost due to premature mortality and disability: DALY) with 77% of the cardiovascular (CV) burden attributed to premature mortality. With regard to specific conditions, coronary heart disease (CHD) was the major contributor to the burden of disease, accounting for 10.2 and 10% of the DALYs in Queensland and Australia, respectively (2). The underlying cause of CVD in the majority of cases is atherosclerosis (3). While great advances have been made in the treatment of CVD, the prevalence of the disease continues to rise. Up to 50% of individuals with CVD do not have any of the traditional CV-risk factors such as hypercholesterolemia, hypertension, smoking, and obesity (4), indicating that other factors must contribute to the disease. Identification of other risk factors for CVD, along with their mechanisms of action, is essential if morbidity and mortality from the disease are to be reduced.

The roles of infection and inflammation in atherosclerosis have become increasingly apparent. Chronic inflammatory periodontal diseases are among the most common human infections with 10–15% of the population experiencing advanced forms of the disease (5, 6). In the context of CVD, individuals with periodontitis are reported to have an increased risk of developing the disease, including coronary artery disease, stroke, myocardial infarction, and atherosclerosis even after adjusting for classical CV-risk factors (7–11). Furthermore, adjusted analysis showed that the bacterial burden of *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Treponema denticola*, and *Tannerella forsythia* in subgingival plaque samples was associated with carotid intima-media thickening (12).

While many studies have shown an association between periodontal disease and CVD, some studies have shown a weak or no association between the two diseases (13–15). The fact that periodontitis and CVD share common risk factors also makes interpretation of the clinical studies complex. Meta-analyses have been conducted, however, and a small but significant association has been demonstrated (16–18). The atherosclerotic susceptible apolipoprotein E deficient (apoE $-/-$) mouse model has provided further support for the role of oral bacteria in atherosclerosis by demonstrating that inoculation with *P. gingivalis* results in advanced atherosclerotic lesions compared with control mice (19–21).

The present review will discuss the current proposed mechanisms linking oral bacteria with CVD, focusing specifically on direct endothelial invasion, systemic inflammation, platelet aggregation, and cross-reactivity between bacterial and host heat shock proteins.

 Citation: Journal of Oral Microbiology 2010, **2:** 5781 - DOI: 10.3402/jom.v2i0.5781

Host–bacterial interactions in periodontal disease

P. gingivalis, *T. denticola*, and *T. forsythia* are part of a small group of Gram-negative anaerobic bacteria that have been found to be involved as pathogens in many periodontitis lesions and have been nominated as the red complex (22). These organisms reside in a complex biofilm that is the dental plaque. While these bacteria are associated with periodontal disease progression, their complex relationships with each other and the many commensal bacteria present in the biofilm must be considered when examining the immune response occurring in periodontal disease. There is considerable variation in the composition of this biofilm that is determined by factors such as the individual, the site examined, and the time of the examination. The biofilm is formed by colonization of the enamel salivary pellicle followed by secondary colonization that occurs by interbacterial adhesion (23). Subgingival plaque, due to its more protected location, is much more resistant to removal than supragingival plaque. Successful colonization of the biofilm by a microorganism can be enhanced by adhesins including fimbriae, hemagglutinins, and proteases (24).

P. gingivalis binds via fimbrillin (*fim*A), the structural subunit of the major fimbriae. It is an asaccharolytic organism and produces hemagglutinins and proteases for its hemin and peptide requirements. The hemagglutinins are also involved in bacterial adherence and the proteases lead to immune responses and tissue destruction (reviewed in Gemmell and Seymour (25)). *P. gingivalis* is associated with deep periodontal pockets and sites of active disease (26). The pathogenicity of this organism is due to several features including fimbriae, which enable its adherence to and invasion of gingival tissues (27), a dense amorphous capsule that allows resistance of phagocytosis, and production of enzymes such as collagenase and trypsin-like protease that facilitates tissue breakdown and degradation of most serum proteins, including immunoglobulins and complement components. *P. gingivalis* also inhibits neutrophil migration into the lesion by its failure to stimulate the expression of the neutrophil binding adhesion molecule E-selectin on gingival endothelial cells and by inhibition of epithelial cell production of interleukin-8 (IL-8). Specific antibodies produced may be ineffective, further inhibiting clearance by neutrophils (reviewed in Gemmell et al. (28)).

A mouse model has been used to define the immune response to *P. gingivalis* (29, 30) and variations in the local T- and B-cell responses occurred with different mouse strains. Interestingly, a study examining coinfection with *P. gingivalis* and *Fusobacterium nucleatum* demonstrated inhibitory effects on antibody production by both bacteria (31). *F. nucleatum* and *P. gingivalis* have been shown to exert differential effects at the molecular level on oral epithelial cells and their differences in activating NK-κB nuclear translocation in oral epithelial cells may at least in part be responsible for the change in dynamics and kinetics of downstream gene expression (32).

Cells of the periodontium, particularly epithelial cells and inflammatory cells interact directly with plaque bacteria and bacterial products. It is the result of these interactions that ultimately determines whether the inflammatory lesion will resolve, become stable, or lead to progressive host tissue destruction. Studies using a coculture model, therefore, have yielded useful information regarding these interactions. A macrophage/epithelial cell coculture model assessing the effect of exposure to mono and mixed preparations of whole cells of red-complex bacteria examined cytokine production at varying multiplicities of infection (MOI). Secretion of IL-1β, IL-6, IL-8, and RANTES was increased and this varied with the bacterial strain and MOI (33).

The ability of red-complex bacteria to suppress innate immune responses of gingival epithelial cells has been demonstrated *in vitro*. Orange-complex bacteria induced strong responses while non-periodontopathic bacteria elicited only weak responses from these cells (production of human beta defensins, the cathelicidin LL-37, and the neutrophil chemoattractant IL-8) (34). Interestingly, while non-periodontopathic and orange-complex bacteria were highly susceptible to the antimicrobial peptides produced and to phagocytosis by neutrophils, red-complex bacteria were resistant. Therefore, non-periodontopathic bacteria may protect the host by activating the gingival epithelium at low levels, orange-complex bacteria may be weakly pathogenic (but have other roles in contributing to a pathogenic biofilm) while red-complex bacteria can effectively evade host immune mechanisms and establish persistent infection.

In contrast to the results of *in vitro* models (33) in which no synergistic effects were observed for mixed bacterial stimulations, there is evidence that periodontopathic bacteria act cooperatively in terms of the development of a pathogenic biofilm and also in causing disease in animal models (35, 36). A number of studies have described the pathogenic synergism between *P. gingivalis* together with *F. nucleatum* (37, 38), *T. denticola* (39), or *T. forsythia* (40). It has been suggested that bacterial cell–cell interactions may enhance both the survival of the organisms in a hostile environment and also their pathogenicity (38).

Virulence factors of oral bacteria

Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria. The LPS is recognized by the innate immune system by means of toll-like receptors (TLRs). TLRs allow recognition of structurally conserved pathogen associated microbial products

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(PAMPs), such as LPS (reviewed by Takeda and Akira (41)). Bacterial LPS is usually recognized by TLR-4; an exception to this is LPS from *P. gingivalis* that, due to a structural change, is recognized by TLR-2 (42). Ligation of TLRs triggers signal transduction pathways leading to a rapid innate inflammatory response and facilitation of antigen specific acquired immunity (reviewed in Takeda and Akira (41)).

CD14 acts as coreceptor with TLR-4 for the detection of bacterial LPS. Increased levels of soluble CD14 in serum of periodontal patients (43) along with increased serum levels of antibodies against LPS of periodontopathogens has been demonstrated (44), suggesting that chronic exposure to bacterial LPS in these patients results in systemic effects. Activation of CD14 leads to the secretion of many proinflammatory molecules including IL-1, IL-6, tumor necrosis factor-alpha (TNF-α), and prostaglandin E2 (45) that, in turn, promotes the release of secondary inflammatory mediators such as platelet activation factor, bradykinin, histamine, and prostaglandins (43, 44, 46, 47).

Bacterial LPS is also known to activate the complement system, a group of more than 30 proteins that participate in tissue destruction and the inflammatory process (45). Activation of the complement system is one of the earliest host immune responses in periodontal infection (reviewed in Genco (48)), and the degree of complement system activation is related to the severity of periodontal inflammation (49). Some oral bacteria are able to avoid opsonization as they exhibit proteolytic activity on their cell surface for degrading components of the complement system (50).

Proteases

Proteases cleave peptide bonds and are characterized according to their catalytic activity. *P. gingivalis* is able to secrete large amounts of proteolytic enzymes. Among the extracellular endopeptidases of *P. gingivalis*, cysteine proteases, termed gingipains (Lys-gingipain, Kgp; Arg-gingipain, Rgp), are major virulence factors. Their activities are essential for bacterial survival but are pathogenic to the host (reviewed in Imamura (51)). Gingipains allow *P. gingivalis* to attach to host tissues by facilitating fimbriae maturation and are also involved in hemoglobin binding for collection of heme and host amino acids, important nutrient sources for the bacteria (reviewed in Potempa et al. (52)) These enzymes are able to degrade a broad range of proteins including collagens (a major component of periodontal connective tissue) (53) and extracellular matrix proteins (fibronectin and laminin) (54, 55), leading to destruction of periodontal tissue (56). Rgp and Kgp also cleave and inactivate immunoglobulins (53) and cytokines (57, 58) thereby compromising host defense mechanisms (52, 59).

As reported by Imamura (51), gingipains also cause decreased IL-6 levels in gingival tissues near the bacterial biofilm, suppressing inflammatory reactions mediated by IL-6 and, therefore, facilitating periodontal tissue destruction. This finding is also confirmed by Oleksy et al. (60), who identified the proteolysis of IL-6 receptors *in vitro* by gingipains from *P. gingivalis*. In addition, other cytokines including IL-8 and IL-1β were found to be cleaved by gingipains (51). Dysregulation of the coagulation cascade (61) and activation of the kallikrein-kinin cascade have also been shown to be caused by Rgp and Kgp (62). Furthermore, Rgp causes increased vascular permeability (51) and produces a chemotactic factor to attract leukocytes to migrate into the periodontal tissues (63). Kgp activates the kallikrein-kinin system in the intrinsic pathway of coagulation leading to the release of bradykinin and prostaglandin thereby promoting alveolar bone resorption (64, 65). Gingipains can also degrade the CD14 receptor for bacterial LPS on the surface of monocytes and T-cells leading to decreased responsiveness to LPS (51, 66, 67). Gingipains have also been shown to stimulate and activate matrix metalloproteinases (MMPs) that destroy periodontal tissue (51). These effects, which lead to enhanced tissue damage, may also help bacteria to enter the blood stream.

A murine abscess model using a *P. gingivalis* strain with a gingipain mutation showed reduced abscess size (40), reduced proteolytic, hemagglutinating, and colonizing activity (68–70), as well as weakened polymorphonuclear leukocyte-suppressive activities (56). Additionally, bacterial clearance was greater in mice injected with *P. gingivalis* gingipain mutants (71). This may be due to the fact that gingipains are important in the maturation of *fim*A, an important component supporting bacterial adhesion and invasion (59, 72). Additionally, lack of gingipains will prevent bacteria from obtaining heme, a nutrition source required for their growth and development (72, 73). Supporting this finding, *P. gingivalis* grown in heme-limiting cultures has been reported to become avirulent in a mouse model (74). Immunization with gingipain in a mouse model protected against further infection by *P. gingivalis* (75, 76).

Fimbriae

Fimbriae are hair-like protein structures protruding from the cell surface of bacteria, particularly Gram-negative bacteria. Fimbriae assist in the binding of bacteria to host cells, as well as in colonization and resisting host defenses (77). *fimA* of *P. gingivalis* has been the most extensively studied and is a critical factor in supporting the initial adhesion and invasion of bacteria to target cells as well as for its colonization on gingival tissues (78, 79). A strain of *P. gingivalis* with mutations in the *fim*A genes showed loss or reduction in adherence or invasion of host cells (80, 81). *In vitro*, *P. gingivalis* has been shown

to invade human epithelial, endothelial, fibroblastic, and periodontal ligament cells (78, 82, 83).

Six genotypes of *fim*A from *P. gingivalis* (types I–V and Ib) have been classified according to their different nucleotide sequence (84–86). Type II *fim*A and type IV *fim*A are believed to be more virulent and have been found to be more prevalent in periodontitis patients with CVD, while type I *fim*A was found mainly in periodontally healthy subjects (87–89). In addition, type IV *P. gingivalis* exhibited higher hemagglutination activity, whereas type II *P. gingivalis* was lower in trypsin-like, arginine-specific activity (BAPNA) (90). In particular, type II *fim*A *P. gingivalis* was reported to cause impaired host cellular function including adhesion, migration, and proliferation as this bacterial strain is able to degrade focal adhesion kinase, paxillin, and integrin-related signaling molecules at significantly greater levels than other strains (78). This strain of *fimA P. gingivalis* was also detected more frequently in deeper periodontal pockets (91) and was the most efficient invader of human epithelial cells compared with other *P. gingivalis* strains (78). Using a mouse abscess model, Nakano et al. (92) showed that among the six strains, *fim*A type II caused the most significant induction of acute general inflammation. Deletion of type II fimbriae led to a depletion in the infection capabilities of the bacterium.

Leukotoxins

Leukotoxins are a group of exotoxins that have primary toxic effects against leukocytes, particularly polymorphonuclear cells (PMNs). Leukotoxin from *A. actinomycetemcomitans* forms pores in the lipid bilayer and is capable of killing human leukocytes via a specific receptor on the cell membrane (reviewed in Narayanan et al. (93)). In human PMNs and macrophages, leukotoxins trigger the translocation of cytoplasmic granules to the periphery, releasing the lysosomal contents, and increasing the oxidative activity leading to inflammation-mediated injury to host tissues (94). Leukotoxin also promotes programmed cell death (apoptosis) by activating endogenous nuclease that leads to an enhanced breakdown of chromosomal DNA; killing PMNs, macrophages, natural killer (NK) cells, and T lymphocytes (95). Importantly, leukotoxin also helps *A. actinomycetemcomitans* to evade the host immune system, allowing the bacterium to establish a local infection. Leukotoxin also acts as a bridge to bind the bacterium to leukocytes, enabling the bacterium to spread throughout host tissues unnoticed (reviewed in Narayanan et al. (93)).

Capsule

Capsules protect bacteria from phagocytosis, aid in cell attachment, and subsequent biofilm formation. Capsular antigens are also targets of the immune system and are involved in cell recognition (96). *P. gingivalis* strains with a capsule are reported to be more virulent and invasive compared with strains without a capsule (78).

Current evidence for the role of oral bacteria in cardiovascular disease (CVD)

While many studies have shown an association between oral infection and CVD, the relationship remains to be established as a causal one. Intervention and pathogenic mechanistic studies are required to strengthen the evidence for the association between the two diseases. A number of mechanisms have been proposed and incorporate both direct and indirect effects of bacteria on the vasculature (97).

A number of reviews have been published outlining the development of atherosclerosis (1, 98, 99). The following discussion will provide a brief overview of the development of atherosclerosis and how oral bacteria may facilitate and enhance this process.

Atherosclerosis is an immune/inflammatory disease and presents as focal thickening of the intima (1). The initiating factor in atherogenesis is endothelial dysfunction. Endothelial dysfunction occurs initially at sites of arterial bifurcations, but risk factors such as hypercholesterolemia and hypertension can also lead to patches of endothelial dysfunction. Low density lipoprotein (LDL) accumulates in the intima and in these areas becomes oxidized (oxLDL). Activated endothelial cells express cellular adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and selectins that mediate rolling and adhesion of circulating leukocytes, such as monocytes and lymphocytes to the endothelial cells. Once adhered to the cell surface, leukocytes migrate into the intima in response to chemoattractant factors such as monocyte chemoattractant protein-1 (MCP-1). Monocytes subsequently differentiate into macrophages, in response to macrophage colony stimulating factor (M-CSF), engulf oxLDL via scavenger receptors, and become foam cells forming the first stage of the atherosclerotic lesion known as the fatty streak (reviewed in Libby (98)).

T-cells (predominately CD4+ cells) present within the intima recognize antigens presented by the major histocompatability complex (MHC) molecules expressed on the surface of antigen presenting cells and mount cell-mediated immune responses. T helper 1 (Th1; cellular) immunity is currently believed to be central in atherogenesis and involves proinflammatory mediators such as interferon-gamma (INF-γ), TNF-α, IL-1, and IL-12. The role of T helper 2 (Th2; humoral) mediated immunity in disease progression is still unclear (reviewed in Hansson et al. (99) and Jawien (100)).

Pro-inflammatory cytokines produced by activated macrophages or T-cells contribute to the local inflammation of the lesion and stimulate the migration and proliferation of smooth muscle cells (SMCs). The

Citation: Journal of Oral Microbiology 2010, 2: 5781 - DOI: 10.3402/jom.v2i0.5781

SMCs subsequently secrete extracellular matrix components that lead to the formation of a fibrous cap over the lesion. As macrophages accumulate more lipid, apoptosis occurs leading to a necrotic core of extracellular lipid deposits that are covered by a fibrous cap (reviewed in Libby (98)). A complication of the lesion is rupture leading to thrombus formation and obstruction of the vessel that can result in clinical signs of atherosclerosis such as myocardial infarction and stroke (1). Vulnerable lesions are generally characterized by a thin fibrous cap, large necrotic lipid core, and numerous inflammatory cells. Stable lesions, on the other hand, have a thick fibrous cap, small necrotic lipid core, and numerous SMCs (reviewed in Libby (98)). Inflammation, therefore, is evident through all stages of lesion development and not only plays a role in lesion development but also in lesion instability and rupture.

The anatomical proximity of the oral biofilm to the periodontal vasculature facilitates the systemic spread of oral bacteria to distant sites from the oral cavity such as the heart (101). The sulcular epithelium is relatively thin and easily disturbed (reviewed in Parahitiyawa et al. (102)). Mastication, oral hygiene procedures (tooth brushing and dental flossing) (103–105), and periodontal procedures (periodontal probing, tooth extractions, removal of subgingival plaque and calculus, as well as surgery) can disturb the sulcular epithelium leading to a bacteremic state (103, 104). In both gingivitis and periodontitis the periodontal vasculature dilates, increasing in surface area, further facilitating bacteremia (reviewed in Parahitiyawa et al. (102)). Individuals with poor oral hygiene are at a higher risk of developing bacteremia during periodontal procedures due to a higher bacterial burden (104).

Direct arterial infection

It is hypothesized that transient bacteremia during periodontal infection may lead to direct bacterial invasion of endothelial cells. Invasion of non-phagocytic cells is a common strategy of pathogens to evade host defenses. Indeed, bacteria, including those of oral origin, have been identified by PCR in atherosclerotic plaques (106–110). Multiple oral bacteria (*Streptococcus mutans*, *Streptococcus sanguinis*, *A. actinomycetemcomitans*, *P. gingivalis*, and *T. denticola*) have been detected in both aortic aneurysm specimens and in heart valve specimens (111). Oral bacteria have also been frequently detected at sites along the arterial tree where atherosclerotic lesions commonly occur. For example, *P. gingivalis*, *A. actinomycetemcomitans*, *Prevotella intermedia*, and *T. forsythia* were commonly found in coronary arteries but not in internal mammary arteries (96). Supporting this finding, Ford et al. (109) identified *P. gingivalis* in 100% of the carotid endarterectomy specimens examined and *F. nucleatum* and *T. forsythia* in 84 and 48% of specimens, respectively. Furthermore, two or more species of periodontal bacteria were detected in 64% of atherosclerotic plaques from CHD patients with periodontal disease (112). It is unclear, however, if the presence of oral bacteria in atherosclerotic lesions contributes to the progression of atherosclerosis or constitutes an innocent bystander effect.

In support of direct effects of oral bacteria on endothelial cells, invasive strains of *P. gingivalis* have been shown to induce their uptake by macrophages and enhance foam cell formation, in the presence of LDL, *in vitro* (113). Furthermore, certain strains of *P. gingivalis*, *P. intermedia* (114, 115), and *S. mutans* (116) have been shown to invade and persist *in vitro* within aortic endothelial cells. Following on from this, *P. gingivalis* has been shown to replicate intracellularly within autophagosomes (117). The ability of *P. gingivalis*, as well as other bacteria, to persist intracellularly may allow the establishment of secondary chronic infections that may further contribute to the pathology of atherosclerosis.

Invasion of endothelial cells by bacteria can lead to endothelial dysfunction, a key event in the development of atherosclerosis. Endothelial dysfunction is associated with increased procoagulant properties, mononuclear cell adhesion, increased expression of cell adhesion molecules, and proinflammatory cytokines and chemokines (such as IL-6, IL-8, and MCP-1) all of which have been shown to be induced by *P. gingivalis* (118, 119). These proinflammatory and proatherogenic effects of *P. gingivalis* have been linked to the invasive capacity of the bacterium as DPG3, a mutant form of *P. gingivalis* lacking major fimbriae, was unable to induce these effects in endothelial cells (118, 119). Bacterial invasion of host cells can also result in apoptosis as high concentrations of *P. gingivalis,* but not DPG3, were able to induce apoptotic cell death of endothelial cells (120). Therefore, an invasion of endothelial cells by oral bacteria may lead to changes in the proinflammatory and proatherogenic properties of endothelial cells as well as programmed cell death, all of which are indicative of endothelial dysfunction.

Platelet aggregation

There is increasing evidence that oral bacteria interact with platelets. While the primary role of platelets is hemostasis, they also play a role in the immune response to infection (121). Oral bacteria may affect platelets directly or indirectly. Firstly, the immune response to oral bacteria may activate platelets; secondly, oral bacteria may secrete products that activate platelets; and thirdly, bacteria may bind to platelets resulting in activation (reviewed in Kerrigan et al. (122)). Activation of platelets by bacteria can lead to localized thrombus formation, platelet consumption, as well as increased secretion of proinflammatory cytokines and mediators from platelets, which further contribute to the pathology of atherosclerosis (reviewed in Fitzgerald et al. (123)).

A number of *Streptococci* species particularly of the viridans group, *S. sanguinis, S. gordonii, S. mutans, and S. mitis,* have been found to induce platelet adhesion and aggregation *in vitro* (reviewed in Kerrigan and Cox (124)). The process is multifactorial and involves many cell surface proteins including: the platelet aggregation associated protein (PAAP) of *S. sanguinis* (125), serine rich glycoproteins designated SrpA and GspB/Hsa in *S. sanguinis* (126) and *S. gordonii* (127), respectively, adhesions such as SspA and SspB in *S. gordonii* (122), as well as glucosyltansferases (128).

Platelet aggregation is not limited to *Streptococci* species as certain strains of *P. gingivalis* also induce platelet aggregation *in vitro*. The platelet aggregating ability of *P. gingivalis* was first attributed to fimbriae as fimbriaed strains of *P. gingivalis* were potent activators of murine platelets and the DPG3 mutant was ineffective at inducing aggregation (129). Purified fimbriae alone, however, was unable to induce platelet aggregation suggesting that other factors, in addition to fimbriae, must also be involved in the process (129). *P. gingivalis* expresses a non-collagen like antigen that is cross-reactive with PAAP of *S. sanguinis* (130); however, further characterization of this antigen has not been carried out to date.

Vesicles of *P. gingivalis* have been shown to induce platelet aggregation (129). Vesicles are outgrowths of the outer membrane and contain many virulence factors such hemagglutinins, proteases, and LPS (131). It is proposed that fimbriae are involved in the binding of the bacterium to platelets and that the binding leads to the interaction of surface expressed vesicle proteins with platelet receptors leading to platelet aggregation (129). Further analysis of the protein vesicles in platelet rich plasma showed that *P. gingivalis* induced platelet aggregation was dependent on cysteine proteases (Kgp and Rgp gingipains) and adhesions such as Hgp44 (132). Recently, *P. gingivalis* induced aggregation has also been shown to occur via a TLR-2 dependent mechanism. To date, other periodontal pathogens have not been observed to induce platelet aggregation (129).

Systemic inflammation

Currently there is strong evidence to support the role of systemic inflammation in the development and progression of atherosclerosis. Epidemiological studies have shown that CV-risk is associated with increased levels of cytokines (IL-1, IL-6, TNF-α, and MCP-1), and acute phase proteins such as C-reactive protein (CRP) and fibrinogen (133–135). Furthermore, plasma levels of inflammatory markers such as CRP have been shown to be a stronger predictor of future CV events than LDL levels, strengthening the importance of inflammation in the progression of atherosclerosis (136). CRP has also been shown to be directly involved in the development of atherosclerosis. CRP stimulates the expression of cell adhesion molecules on arterial endothelial cells, induces the recruitment of monocytes into the arterial wall, and stimulates LDL uptake by macrophages, thereby promoting foam cell formation (reviewed in Libby (98)).

As outlined earlier, activated endothelial cells express cellular adhesion molecules. These molecules can be shed from the cell surface and soluble forms are markers of endothelial activation. Indeed, elevated levels of soluble (s) ICAM-1, sVCAM-1, and selectins have been associated with CV-risk (137).

In relation to the systemic inflammation model it is hypothesized that cytokines and inflammatory mediators produced in periodontal disease act systemically to promote systemic disease. In addition, the release of bacteria and bacterial by-products such as LPS into the circulation may also trigger a systemic inflammatory response. Increased levels of circulating cytokines can activate vascular endothelial cells, ultimately leading to the development of atherosclerosis. In addition to activating inflammatory cells, bacterial LPS may trigger and enhance atherosclerosis by increasing oxidative stress and modifying lipid metabolism (138).

A number of studies showed that patients with severe periodontitis have increased levels of systemic inflammatory markers (IL-6, CRP, haptoglobin, and leukocytosis) (139–144). In addition, periodontitis patients were also found to exhibit dyslipidemia (145) and endothelial dysfunction as assessed by flow-mediated dilatation of the brachial artery (140).

Periodontal intervention studies have been implemented to further investigate the contribution of periodontal infection to systemic markers of inflammation and vascular health. In the short-term (hours to 1 week), periodontal therapy appears to elicit a systemic inflammatory response and mediate endothelial activation/dysfunction as a result of transient bacteremia immediately following therapy (146–148). In the long-term, endothelial function improves and is evident up to 6 months post-therapy (148, 149).

The long-term effects of periodontal therapy in relation to systemic cytokine levels are inconsistent among studies, especially with regard to the cytokines affected and the time frame at which the effect was observed. Patients receiving periodontal therapy in conjunction with systemic administration of antibiotics exhibited no significant change in CRP, IL-6, or TNF-α following a 3 month period (150). While Buhlin et al. (151) also failed to detect changes in markers of inflammation at the same time point, levels of IL-8, INF-γ, and haptoglobin were reduced and lipid profiles improved 12 months after conventional periodontal therapy. In contrast, periodontitis patients receiving intensive periodontal therapy (standard periodontal therapy plus local administration of minocycline) had significant reductions in IL-6, CRP, lipid markers, and systolic blood pressure (SBP) 2 months post-therapy

Citation: Journal of Oral Microbiology 2010, 2: 5781 - DOI: 10.3402/jom.v2i0.5781

compared with patients who received standard therapy alone. In addition, patients receiving intensive periodontal therapy also displayed reductions in Framingham CV-risk scores 2 and 6 months following therapy (152). In patients with hypertension followed for 3 months, non-surgical periodontal therapy resulted in reduced levels of CRP, IL-6, and fibrinogen (153). Furthermore, while levels of CRP, IL-6, and fibrinogen were reduced in patients with and without CHD, changes in fibrinogen and white blood cell count were higher in patients with CHD (154).

Tonetti et al. (148) demonstrated an improvement in endothelial dysfunction and reduced levels of sE-selectin and neutrophil counts 6 months post-therapy; however, the study failed to detect differences in CRP, IL-6, and plasminogen activator inhibitor-1 (PAI-1) between treatment and control groups at the same time point. Periodontal therapy, including surgery, reduced levels of vascular health (PAI-1, sE-selectin, sVCAM-1, MMP-9, and myeloidperoxidase) in patients with periodontitis; however, only reduced levels of sE-selectin were maintained 1 month after the completion of the periodontal therapy regime (155). A summary inflammation score used to assess the systemic inflammatory burden of each patient indicated that there was a high degree of patient variability in the systemic inflammatory response to therapy. Thirty-three percent of patients experienced a reduction in the inflammatory score, 25% experienced an increase in the inflammatory score, while the remainder of the patients experienced no change in the inflammatory score following therapy (155). The apparent patient variability in systemic inflammation may be due to host genetic factors. Systemic inflammatory responses are higher in periodontitis patients carrying alleles for functional polymorphisms in IL-1α, IL-6, and TNF-α genes (156) and these individuals may be more likely to experience anti-inflammatory effects after periodontal therapy.

Another marker of chronic inflammation is anemia. Anemia of chronic diseases (ACD), as it is otherwise known, is a cytokine-mediated anemia that is characterized by impaired mobilization of iron from reticuloendothelial stores, reduced red blood cell survival, and impaired erythropoiesis. Cytokines that have been implicated in ACD include IL-1, IL-6, TNF-α, and INF-γ (reviewed in Means (157)). Periodontal infections have been associated with anemia of chronic inflammation. Periodontitis patients have been shown to exhibit an anemic state as indicated by reduced hematocrit levels, decreased erythrocyte numbers, and reduced hemoglobin levels (158, 159). Intervention studies have further supported this association demonstrating that non-surgical periodontal therapy can result in significant increases in hemoglobin and hematocrit levels within treatment groups (159) as well as compared with control patients who received no periodontal therapy (160).

While there are inconsistencies among studies, the general trend is that periodontal therapy can improve systemic markers of inflammation and vascular health; however, the long-term effects of this with regards to CV events remain unknown. It is difficult to interpret the results of the intervention studies due to differences in periodontal therapy (including duration), time frame at which samples were assessed, and the exclusion of control, non-intervention, groups in some studies. A recent meta-analysis of six studies investigating the effects of periodontal therapy on CRP has provided further insight into the strength of the relationship, and concluded a modest association between periodontal therapy and reduced levels of CRP (161).

The reduction in inflammatory markers after periodontal therapy provides evidence that the elevated levels of systemic inflammation were due to periodontal disease rather than other inflammatory conditions and supports the hypothesis that periodontal inflammation may add to the systemic inflammatory burden of affected individuals.

Cross-reactivity

There is increasing evidence that immune responses may also be involved in the progression of atherosclerosis (97). One mechanism in which infection may initiate and facilitate the progression of atherosclerotic lesions can be explained in terms of the immune response to bacterial HSPs, termed GroEL (162).

HSPs are expressed by prokaryotic and eukaryotic cells in response to various forms of stress (temperature, mechanical stress, infection, and oxygen radicals) (163). They are highly conserved throughout nature and many pathogens express HSPs that are homologous to human HSPs (164). During infection, bacterial HSPs are highly immunogenic (165). Factors such as mechanical stress, bacterial LPS, and cytokines may induce the expression of host protective HSP60 (hHSP60) on endothelial cells.

Due to the homologous nature of HSPs across species, the immune system may not be able to differentiate between host and bacterial HSPs. As a result of this molecular mimicry, there may be cross-reactivity of the immune response to bacterial HSPs with HSPs expressed by stressed endothelial cells leading to endothelial dysfunction and subsequent development of atherosclerosis (162). The presence of CV-risk factors such as hypertension and hypercholesterolemia would enhance the expression of hHSP60 and adhesion molecules by endothelial cells and result in progression from early fatty streak lesions to more severe and irreversible atherosclerotic alterations. Supporting this, Perschinka et al. (166) identified that hHSP60 was expressed to varying degrees in healthy arteries and atherosclerotic lesions.

Elevated levels of antibodies to hHSP60 have been associated with the presence and severity of coronary

artery disease (167) and atherosclerosis (168). In particular, Metzler et al. (168) demonstrated a correlation between high anti-HSP60/65 antibody levels and high morbidity and mortality due to atherosclerosis. These antibodies were cross-reactive with other bacterial HSPs and were able to lyse stressed – but not unstressed – endothelial cells (169). Elevated anti-hHSP60 levels have also been found in patients with carotid stiffness (170), borderline hypertension, and have been associated with early atherosclerosis (171).

The mechanism by which antibodies to self-antigens, such as hHSP60, are generated is unclear. It is currently proposed that anti-hHSP60 antibodies could represent a cross-reactive host immune response to bacterial HSPs in susceptible individuals or result from a primary immune response to altered self-HSP60 expression (172).

The GroEL proteins are major antigens in several pathogenic bacterial (173). GroEL proteins and homologues have been identified in several oral bacteria including *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum*, *P. intermedia*, *T. denticola*, and *S. mutans* (reviewed in Goulhen et al. (174)). GroEL from *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemcomitans* was recognized by serum antibodies in patients with periodontal disease (175). Patients with periodontal disease have been shown to have a higher antibody response to hHSP60 and *P. gingivalis* GroEL compared with periodontally healthy controls. In addition, these antibodies were cross-reactive with *P. gingivalis* GroEL and hHSP60, respectively (176).

Due to the homologous nature of bacterial GroEL with hHSP60, periodontal infections have been associated with atherosclerosis possibly due to cross-reactivity of the immune response to bacterial GroEL with hHSP60. Previous studies have demonstrated the presence and cross-reactivity of hHSP60, *P. gingivalis* GroEL, and *P. gingivalis* specific T-cells in the gingival tissues of chronic periodontitis patients (177), in the peripheral blood of atherosclerotic patients, and in human atherosclerotic lesions (178). Furthermore, antibodies to *P. gingivalis* from atherosclerotic patients with periodontitis cross-reacted with hHSP60 (109). In an intervention study, Yamazaki et al. (179) found that periodontal therapy significantly reduced the levels of anti-*P. gingivalis* GroEL antibodies in patients with moderate to advanced periodontal disease, while the mean levels of antibodies to hHSP60 remained unchanged. Similarly, Buhlin et al. (151) detected no change in anti-hHSP60 antibody levels following periodontal therapy.

Murine models of atherosclerosis have also provided evidence for the role of HSP immunity in atherosclerosis. Immunization of C57BL/6 mice fed a cholesterol rich diet with HSP resulted in enhanced atherosclerosis (180). Furthermore, an injection of human anti-hHSP60 antibodies in apoE ($-/-$) mice enhanced atherosclerosis (181). This is supported by an intervention study that demonstrated protection from atherosclerosis following oral tolerance to HSP60 (182). In addition, Ford et al. (21) showed a significant correlation between anti-GroEL antibody levels and atherosclerotic lesion size in apoE ($-/-$) mice inoculated with *P. gingivalis,* indicating that molecular mimicry may be a mechanism involved in infection-induced atherosclerosis.

Conclusion

There is clear evidence of an epidemiological association between oral infections and CVD and *in vitro* and *in vivo* mechanistic studies have established a plausible link between oral bacteria and atherosclerosis. The interactions between oral bacteria and CVD are extremely complex and it's highly likely that more than one mechanism is involved.

Recent studies provide strong support for the roles of systemic inflammation and immune cross-reactivity in atherogenesis. Furthermore, intervention studies have demonstrated that periodontal therapy can reduce systemic markers of inflammation and vascular health as well as antibody responses to heat shock proteins. Future studies are now required to determine the long-term effects of periodontal therapy on CVD outcomes. Nevertheless, a recent consensus report on periodontitis and atherosclerotic CVD, published in the *American Journal of Cardiology* and the *Journal of Periodontology* (183), has recommended that patients with moderate to severe periodontitis should be informed of a possible increased risk of CVD and those with more than one CV-risk factor should undergo a medical evaluation of CV-risk.

Conflict of interest and funding

There is no conflict of interest in the present study for any of the authors.

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Citation: Journal of Oral Microbiology 2010, 2: 5781 - DOI: 10.3402/jom.v2i0.5781

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Citation: Journal of Oral Microbiology 2010, **2**: 5781 - DOI: 10.3402/jom.v2i0.5781

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