ORAL DISEASES

Oral Diseases (2011) 17, 450–461 doi:10.1111/j.1601-0825.2010.01784.x

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INVITED MEDICAL REVIEW

Mechanisms involved in the association between peridontal diseases and cardiovascular disease

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It is now well accepted that besides the cholesterol associated mechanisms of atherogenesis, inflammation plays a crucial role in all stages of the development of the atherosclerotic lesion. This 'inflammation hypothesis' raises the possibility that through systemic elevations of pro-inflammatory cytokines, periodontal diseases might also contribute to systemic inflammation and, therefore, to atherogenesis. In fact, there is evidence that periodontal diseases are associated with higher systemic levels of high-sensitivity C-reactive protein and a low grade systemic inflammation. This phenomenon has been explained based on mechanisms associated with either the infectious or the inflammatory nature of periodontal diseases. The purposes of this article were to review (1) the evidence suggesting a role for oral bacterial species, particularly periodontal pathogens, in atherogenesis; (2) the potential mechanisms explaining an etiological role for oral bacteria in atherosclerosis; (3) the evidence suggesting that periodontal infections are accompanied by a heightened state of systemic inflammation; (4) the potential sources of systemic inflammatory biomarkers associated with periodontal diseases; and (5) the effects of periodontal therapy on systemic inflammatory biomarkers and cardiovascular risk.
Oral Diseases (2011) **17**, 450–461

Keywords: cardiovascular disease; atherosclerosis; periodontal diseases; infection; periodontal pathogens; bacteremia; inflammatory response; systemic biomarkers; C-reactive protein

Introduction

Since Mattila *et al* (Mattila *et al*, 1989) reported the first study examining the association between cardiovascular disease (CVD) and dental infections in 1989, the literature has been flooded with reports examining the strength of this association and its biological plausibility. Several epidemiological studies have been conducted examining a possible association between periodontal disease and CVD (DeStefano *et al*, 1993; Mattila *et al*, 1995; Beck *et al*, 1996; Joshipura *et al*, 1996; Morrison *et al*, 1999; Hujoel *et al*, 2000; Wu *et al*, 2000; Howell *et al*, 2001; Ajwani *et al*, 2003; Tuominen *et al*, 2003; Hung *et al*, 2004) and three meta-analyses have summarized their findings (Janket *et al*, 2003; Mustapha *et al*, 2007) (Humphrey *et al*, 2008). These meta-analyses have consistently concluded that the available evidence indicates that periodontal diseases confer a moderate risk for atherosclerosis and its consequences (Janket *et al*, 2003; Mustapha *et al*, 2007; Humphrey *et al*, 2008).

Because periodontal diseases are infections/inflammatory diseases, mechanisms mediated by oral microorganisms and the inflammation triggered by them have been proposed to explain their involvement in atherogenesis. Bacteria associated with periodontal diseases can colonize the atheromatous plaques and could cause their damage by inducing local inflammation, resulting in propagation of the inflammatory events that lead to atheroma formation, development and eventual rupture. Alternatively, a low grade systemic inflammation could result from bacteremias, or as a consequence of pro-inflammatory cytokines generated at the site of the periodontal lesion gaining access to the blood stream. In fact, the potential role of infection in the etiology of CVD is not new and over the years several different microbial agents have been investigated for their role in atherosclerosis. Similarly, diseases associated with a heightened state of systemic inflammation such as rheumatoid arthritis (RA) have also been associated with atherosclerosis and its acute complications. The purpose of this manuscript was to review the infectious and inflammatory mechanisms proposed to explain the link between periodontal diseases and CVD, and to appraise the evidence supporting them.

Inflammatory mechanisms of atherogenesis

The normal endothelium is non-adherent to circulating leukocytes. Once exposed to inflammatory stimuli,

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Received 2 November 2010; Revised 5 November 2010; accepted 5 November 2010

endothelial cells increase the expression of adhesion molecules such as inter-cellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and P-selectins and beta2 integrins, allowing for the firm attachment of leukocytes (Libby and Theroux, 2005). Endothelial activation/dysfunction is a first step in the establishment of the incipient atherosclerotic lesion. Under inflammatory conditions, endothelial cells will also secrete chemokines such as monocyte chemotactic protein-1 (MCP-1) which directs the migration of monocytes (Boring *et al*, 1998). After adhering to the endothelium, leukocytes will migrate into the intima of the blood vessel wall where they multiply. This migration and diapedesis is facilitated by the expression of matrix metalloproteinases (MMPs) such as MMP-9 by the adherent monocytes (Amorino and Hoover, 1998). Once within the intima, monocytes are induced by macrophage colony stimulating factor (M-CSF) to mature into macrophages (Clinton and Libby, 1992). Macrophages will also respond to M-CSF and express scavenger receptors, which engulf through endocytosis lypoproteins modified by inflammation (Packard and Libby, 2008). Accumulation of oxidized low-density lipoprotein (LDL) in the cytoplasma of macrophages will result in the formation of the so-called foam cells, characteristics of fatty streaks. At the same time, macrophages release an array of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) amplifying the local inflammatory response and the events that lead to the early-stages of atherosclerosis. Apoptosis of foam cells will result in the release and accumulation of lipids in the intima (Packard and Libby, 2008).

The cytokines generated in the atherosclerotic lesions induce primarily a T helper 1 cell (Th1) response, which, in turn, amplifies the local inflammation (Robertson and Hansson, 2006). Atheromas grow in shoulder regions of the atherosclerotic lesions where macrophages and T cells infiltrate and deposition of fibrous tissues occurs (Packard and Libby, 2008). Smooth muscle cells (SMCs) migrate from the tunica media into the intima after degradation of the extracellular matrix by MMP-9 and other proteases (Mason *et al*, 1999). Once in the intima, SMCs proliferate under the influence of various growth factors such as transforming growth factor beta (TGFβ) and platelet-derived growth factor (PDGF) and secrete collagen and other extracellular matrix components. This phase of the atheroma formation is characterized by its change from a lipid-rich plaque to a fibrous plaque, leading to stenosis (Packard and Libby, 2008).

The key histological features of the site of the atheroma rupture are the presence of large numbers of macrophages and a paucity of SMCs. There are several inflammatory mechanisms that might lead to fibrous cap rupture. The overall effect of inflammation on extracellular matrix components is the stimulation of their degradation and inhibition of their synthesis. IFNγ can inhibit collagen production, and intersticial collagenases including MMP-1, MMP-8 and MMP-13 can be released by macrophages upon stimulation by pro-inflammatory signals such as IL-1β and CD40L. The shoulder region and areas of foam cells contain MMP-9 and active MMP-9 can be recovered from human atherosclerotic plaques. These effects decrease the collagen content of the fibrous cap rendering it susceptible to rupture. Fibrous cap rupture exposes the atherosclerotic lesion to the liquid phase of blood, releasing a series of pro-coagulant factors, triggering thrombus formation and leading to obstruction of the blood flow (Haraszthy *et al*, 2000; Packard and Libby, 2008).

The oral infectious theory of atherogenesis

Detection of oral species in atherosclerotic lesions

To explore the possibility that oral bacteria could be associated with atherosclerosis, several investigators have examined if molecular signatures of oral species could be found in atheromatous lesions. These studies have utilized different techniques including polymerase chain reaction (PCR) of 16S rRNA genes (Haraszthy *et al*, 2000; Fiehn *et al*, 2005; Pucar *et al*, 2007; Aimetti *et al*, 2007; Mahendra *et al*, 2010), real-time PCR (Kozarov *et al*, 2006; Gaetti-Jardim *et al*, 2009), DNA-DNA hybridization (Elkaim *et al*, 2008), fluorescence in situ hybridization (FISH) (Cavrini *et al*, 2005) and culture of periodontal pathogens from atheromatous plaques (Kozarov *et al*, 2005). Although there is some logic in examining periodontal pathogens, as they present several characteristics that make them particularly attractive as causative agents of inflammatory processes, it is also quite conceivable that the properties required by an oral species to induce atherosclerosis differ considerably from those involved in periodontal tissue destruction. For instance, infective endocarditis is caused primarily by viridans group *streptococci*, *staphylococci*, and *enterococci*, none of which are putative periodontal pathogens (Baddour *et al*, 2005). In fact, an array of other oral species might be recovered from atheromas, including the etiological agent of dental caries, *Streptococcus mutans* (Nakano *et al*, 2006). In addition, bacteremia studies have revealed a large diversity in oral species entering the blood stream after different bacteremic stimuli, ranging from *streptococci* species to anaerobic gram-negative bacteria (Forner *et al*, 2006a).

Mechanisms of oral bacteria induced atherosclerosis

Endothelial cell invasion. The concept that infection can contribute to atherosclerosis is based primarily on studies examining pathogens that can cause persistent infection with the pathogen residing in cells for prolonged periods of times without proliferating. Agents such as cytomegalovirus (CMV), herpes simplex virus (HSV) and *Chlamydia pneumoniae* that have been implicated in human atherosclerosis by seroepidemiological studies are all intracellular pathogens (Epstein *et al*, 2009). Therefore, when examining potential pathological mechanisms linking periodontal diseases to atherosclerosis, it seems logical to explore host cell invasiveness by oral species. Results from a pioneer study by Deshpande *et al* (Deshpande *et al*, 1998) demonstrated that *P. gingivalis* could invade endothelial

cells *in vitro* and that the expression of fimbriae was necessary for this process. A study published soon after this initial report (Dorn *et al*, 1999) indicated that this phenomenon was not only species but also strain specific. A fimbriae expressing strain of *P. gingivalis* (381) had a higher capacity of invasion than the strain without fimbriae on its surface (W50), a strain of *P. intermedia* was invasive while another one was not, and *Eikenella. corrodens* presented a minimal ability to invade. Since these early reports, several other oral species including *Porphyromonas endodontalis* (Deshpande *et al*, 1998), *S. mutans* (Abranches *et al*, 2009), *Streptococcus gordonii*, *Streptococcus sanguinis*, *Streptococcus mitis*, and *Streptococcus oralis* (Stinson *et al*, 2003) have been shown to be able to invade endothelial cells.

The importance of invasiveness was highlighted in a study where, using high-density oligonucleotide microarrays, the authors examined the gene expression profile of human aortic endothelial cells (HAEC) after infection with invasive and non-invasive strains of *P. gingivalis*. Infection of HAEC with invasive *P. gingivalis* strain 381 resulted in the upregulation of 68 genes. Genes coding for the pro-inflammatory cytokines, adhesion molecules, chemokines and cyclooxygenase-2 were among the most highly upregulated genes. Only 4 of these 68 genes were also upregulated in HAEC infected with the non-invasive *P. gingivalis* fimA mutant. Additional studies using immune techniques confirmed the expression of ICAM-1, VCAM-1, E-/P-selectins, IL-6, and IL-8 in HAEC infected with invasive *P. gingivalis* (Chou *et al*, 2005). Invasion of HAEC with *P. gingivalis* can also induce procoagulant effects including enhanced tissue factor expression and activity, and suppression of tissue factor pathway inhibitor. In addition, infection with invasive *P. gingivalis* 381 decreased levels and activity of tissue plasminogen activator and enhanced expression and activity of plasminogen activator inhibitor-1. Conversely, infection with a non-invasive strain of *P. gingivalis* failed to induce these procoagulant effects in HAEC (Roth *et al*, 2006).

It has also been suggested that certain species can help in the invasiveness of others. It was demonstrated that co-infection with *F. nucleatum* resulted in 2–20-fold increase in the invasion of endothelial cells by *P. gingivalis* strains, highlighting the importance of mixed infection in the modulation of the invasiveness of *P. gingivalis* (Saito *et al*, 2008). A recent study has suggested that after a prolonged intracellular phase *P. gingivalis* loses its ability to multiply, but upon co-incubation with fresh vascular host cells, it could be recovered. The data indicated that intercellular transmission could rescue latent intracellular *P. gingivalis* from a state of dormancy to a viable state (Li *et al*, 2008). In addition, the use of antibiotics that block the invasiveness of *P. gingivalis* has been shown in a murine model to decrease the systemic cytokine response after inoculation of *P. gingivalis* (Amar *et al*, 2009). These lines of evidence clearly indicate a potential role for endothelial cell invasion by oral species as a pathogenic mechanism in atherogenesis.

Endothelial activation. As described earlier, endothelial activation and the increased expression of adhesion molecules and chemokines are the first steps in the development of atherosclerotic lesions. *A. actinomycetemcomitans* infection of apolipoprotein E-deficient mice with a hyperlipidemic phenotype resulted in increased expression in the aorta of ICAM-1, E-selectin, P-selectin, MCP-1, chemokine (C-C motif) ligand 19 (CCL19), CCL21, and CCR7. Coculture of endothelial cells with *P. gingivalis* strains also resulted in the expression of ICAM-1, VCAM-1 and P- and E-selectins in a cell invasion-dependant mechanism mediated by fimbriae (Khlgatian *et al*, 2002; Takahashi *et al*, 2006). Other studies also reported that *P. gingivalis* can induce endothelial cells to express MCP-1. Dead *P. gingivalis* cells could still induce MCP-1 but at much lower levels. Chemical inhibition of endocytosis blocked MCP-1 upregulation, indicating the need for *P. gingivalis* internalization for MCP-1 stimulation (Kang and Kuramitsu, 2002).

By contrast, stimulation of endothelial cells with lipopolysacharide (LPS), outer membrane protein and heat shock protein 60 derived from *P. gingivalis* had only mild effects on the expression of ICAM-1 and VCAM-1 (Honda *et al*, 2005). Treatment of endothelial cells with *P. gingivalis* gingipains results in loss of adhesion properties and apoptotic cell death (Sheets *et al*, 2005). Paradoxically, invasion of endothelial cells by live *P. gingivalis* resulted in downregulation of IL-8 and MCP-1 through a lysine-specific cysteine proteinase (gingipain K)-mediated mechanism (Nassar *et al*, 2002). Another report by the same group demonstrated that invasive strains of *P. gingivalis* resulted in upregulation of IL-8 and MCP-1 (Takahashi *et al*, 2006). Soluble products from *Eikenella corrodens* can induce ICAM-1, VCAM-1, E-selectin and IL-8. Furthermore, these soluble products were capable of upregulating gene expression and protein production of vascular endothelial growth factor (VEGF) by human endothelial cells and activating endothelial cell proliferation (Yumoto *et al*, 2007).

Toll-like receptors mediated mechanisms. Exposure of endothelial cells to oral species might also result in increased expression and interactions with Toll-like receptors (TLRs). *A. actinomycetemcomitans* infection of apolipoprotein E-deficient mice resulted in increased expression in the aorta of TLR2 and TLR4 (Zhang *et al* 2010). Invasive strains of *P. gingivalis* can also stimulate the expression of TLR2 and TLR 4 on the surface of endothelial cells, while non-invasive, fimbriae deficient mutants, failed to do so (Yumoto *et al*, 2005). Extended exposure of LPS from *P. gingivalis* facilitates mononuclear cell adhesion to vascular endothelium via TLR2 mechanisms *in vitro* (Nakamura *et al*, 2008). Endothelial cells incubated with *P. gingivalis* LPS expressed ICAM-1 and VCAM-1 and antibodies against TLR2 and ICAM-1 blocked the enhanced mononuclear cell adhesion (Nakamura *et al*, 2008). Pro-inflammatory cytokine induction by *P. gingivalis* fimbriae can also be inhibited by monoclonal antibodies to TLR2, TLR4, CD14 and beta2 inregrins. Similarly, cytokine induction by

T. forsythia protein A requires CD14 and TLR2. Antigen I/II from *S. mutans* can also stimulate cytokines partially through interactions with CD14 and TLR4 (Hajishengallis *et al*, 2002).

Autoimmunity. There is considerable evidence indicating that auto-immune mechanisms are involved in atherosclerosis. Surface expression of heat shock proteins (HSPs) as a consequence of inflammation, infection and oxidizing agents may be perceived as 'cryptic antigens' and recognized as 'foreign' by the immune system. In fact, in human atherosclerotic lesions, endothelial cells, macrophages and SMCs all express human heat shock protein 60 (hHSP60) and serum levels of anti-hHSP60 correlate with the presence and extent of CVDs (Epstein *et al*, 2009). Cross-reaction of the immune response to bacterial GroEL with HSP60 has been suggested as a pathogenic mechanism linking oral species to atherosclerosis. As hHSP60 and the bacterial GroEL are highly conserved, a cross-reaction between antibodies to GroEL might lead to endothelial activation and damage (Ford *et al*, 2005b). This is an example of how 'molecular mimicry' can contribute to atherogenesis (Epstein *et al*, 2009). Levels of antibodies to GroEL and *P. gingivalis* could be reduced in plasma samples from subjects with atherosclerosis using absorption with human HSP60, suggesting cross-reactivity among these antigens (Ford *et al*, 2005b). In addition, GroEL-, HSP60-, and *P. gingivalis*-specific T-cell lines from atherosclerotic plaques have also demonstrated cross-reactivity (Ford *et al*, 2005a). Using immunohistology, the same group demonstrated the expression of hHSP60 in endothelial cells, smooth muscle cells and lymphocytes from carotid specimens, while GroEL was detected in intima cells (Ford *et al*, 2006).

Metalloproteinase and oxidative stress mediated mechanisms. Oral bacterial species may also induce the upregulation of MMPs by endothelial cells. Increased expression of MMP-9 in the aorta and a proatherogenic lipoprotein profile (smaller particles sizes in VLDL, LDL, and HDL lipoprotein fractions) in Apo-E deficient mice infected with *A. actinomycetemcomitans* has also been reported (Tuomainen *et al*, 2008). Oxidation of LDL is essential for its accumulation within macrophages and the formation of foam cells, can upregulate pro-atherogenic chemokines and adhesion molecules (Li *et al*, 2003) and induce the secretion of IL-6, TNF-α, and C-reactive protein (CRP) (Hulthe and Fagerberg, 2002). *P. gingivalis* can also stimulate LDL oxidation (Bengtsson *et al*, 2008) and rupture of atherosclerotic plaque through induction of MMPs (Ding *et al*, 1995). Co-incubation of a murine macrophage cell line with *P. gingivalis*, *P. gingivalis* outer membrane vesicles, and its LPS in the presence of LDL resulted in the formation of foam cells in a dose dependant manner (Qi *et al*, 2003). Furthermore, fibrous cap material isolated from atheromatous lesions obtained from human autopsy can be degraded by *P. gingivalis in vitro* (Kuramitsu *et al*, 2001).

Assessment of exposure

Periodontal inflammation results in an increased tendency for gingival bleeding, an ulcerated pocket epithelium and a larger number of oral bacteria accumulated within the gingival crevice. These conditions might increase the frequency and magnitude of bacteremias in subjects with gingivitis and/or periodontitis. The cumulative area of ulcerated epithelium for all periodontal lesions has been calculated to be 8–20 cm^2 (Hujoel *et al*, 2001). There is also evidence that the incidence and magnitude of bacteremias after chewing, tooth brushing and subgingival scaling increased with the severity of periodontal inflammation (Forner *et al*, 2006a). Forner *et al* also demonstrated that periodontitis subjects presented a larger variety of species in the positive blood samples, reflecting the increased complexity of the subgingival biofilm in these subjects and, possibly, a higher pathogenic exposure (Forner *et al*, 2006a). Therefore, if chronic low-grade systemic exposures to oral microorganisms are associated with an increased risk of atherosclerosis, the severity of the periodontal condition of subjects should correlate with increased risk of CVD.

However, recent studies have questioned the correlation between severity of clinical parameters of periodontitis and increased susceptibility to bacteremias. Lockhart *et al* demonstrated that oral hygiene and gingivitis bleeding were significantly associated with the detection of bacteria in blood samples after tooth brushing, while parameters of periodontitis did not correlate with the incidence of bacteremia (Lockhart *et al*, 2009). These findings suggest that bleeding tendency was a better parameter to estimate the risk of bacteremia after tooth brushing than parameters of periodontal tissue destruction. A study examining the prevalence of bacteremia after flossing in 30 chronic periodontitis and 30 periodontally healthy subjects could not find a difference in the percentage of bacteremia positive subjects between groups (Crasta *et al*, 2009). Third molar extractions have also been associated with a high prevalence of bacteremia, independently of the oral health status of the extracted teeth (Tomas *et al*, 2007). However, a limitation of studies examining only the prevalence of bacteremia is that they do not take into account the intensity of the exposure. This is important because the intensity of the bacteremia is directly correlated with the risk of sequelae. For instance, bacteremia detected in clinical conditions involves levels from 10 to 100 colony forming units (CFU) ml^{-1}, while the level detected during bacterial endocarditis is approximately 200 CFU ml^{-1} (Lucas and Roberts, 2000). Therefore, if the intensity and complexity of bacteremias are relevant for the risk of atherogenesis, studies that do not quantify these parameters should be interpreted with caution.

The appropriateness of clinical parameters of periodontal diseases as a measure of exposure to oral infectious agents leading to CVD has also been challenged by others. Beck and Offenbacker (Beck and Offenbacher, 2002) reported that clinical attachment loss had a weaker association with systemic biomarkers

of inflammation than probing depth or bleeding on probing. In a subsequent study, the same group examined a subset of participants in the Atherosclerosis Risk in Communities (ARIC) Study of 5,002 individuals. They could not find a correlation between the periodontal status of study subjects and the prevalence of coronary heart disease (Beck *et al*, 2005a). These lines of evidence have led researchers to question what would be the appropriate measure of exposure to investigate the link between periodontal infections and CVD.

There are epidemiological studies that have used direct assessments of colonization by periodontal bacteria demonstrating associations between levels of colonization by specific oral bacterial species and CVD outcomes (Desvarieux *et al*, 2005; Renvert *et al*, 2006; Spahr *et al*, 2006; Nonnenmacher *et al*, 2007). Studies examining the correlations between oral microorganisms and CVD are particularly relevant if the link between periodontal diseases and CVD is to be supported on the grounds of an infectious mechanisms. In that context, quantifying the exposure to oral species might be more significant than the assessment of clinical manifestations of oral infections, particularly when certain clinical parameters might be poor surrogates for infection exposure. However, direct measurement of levels of oral microorganisms in the mouth might still not be the best measure of exposure. The current understanding is that oral bacteria could lead to atherogenesis once they have entered the blood stream through bacteremias. Direct assessment of the presence of oral species in the blood is also counter productive because bacteremias are short lived and peak between 30 s and 2 min and decreases considerably after 10 min (Forner *et al*, 2006a; Tomas *et al*, 2007).

Serum antibodies specific to oral bacteria have also been used to assess exposure to an oral infectious challenge (Pussinen *et al*, 2004, 2005, 2007; Beck *et al*, 2005a; Johansson *et al*, 2005). Although results were not always consistent, a meta-analysis of seroepidemiological studies demonstrated that elevated systemic antibody responses to oral pathogens were strongly associated with an increased risk of coronary heart disease (Mustapha *et al*, 2007). For instance, Pussinen *et al* demonstrated in a sample of 893 Finnish subjects free of CVD at baseline and followed for 15 years that the presence of serum antibodies to *P. gingivalis* increased the risk of stroke. High serum antibody levels to *P. gingivalis* predicted stroke with ORs of 1.6 and 2.3 for males and females (Pussinen *et al*, 2007). In their analysis of 5002 subjects enrolled in the ARIC study, Beck *et al* could not establish a correlation between clinical parameters of periodontitis and coronary heart disease (CHD), but demonstrated an association between systemic antibody responses to several oral species and the prevalence of CHD. They concluded that 'the quality and quantity of the host response to oral bacteria may be an exposure more relevant to systemic atherothrombotic coronary events than clinical measures' (Beck *et al*, 2005b).

The use of systemic antibody responses to oral microorganisms can be an attractive measure of infectious exposure for several reasons: 1) the presence of systemic antibodies to a given bacterium indicates systemic exposure to the microorganism or its components (Beck *et al*, 2005a); 2) specific antibodies to a microorganism indicates that the host recognized it as foreign and the level of response can be interpreted as a measure of its pathogenic potential (Haffajee and Socransky, 1994); 3) a vigorous antibody reaction might reflect the level of immune responsiveness of the host, which might also correlate with the increased risk of CVD (Mattila *et al*, 2005); 4) fluctuations in specific antibody levels correlate to infection and parallel changes in the burden of a specific microorganism in the subgingival plaque (Ebersole *et al*, 1992); 5) systemic antibody levels decrease after periodontal therapy, in response to a decrease in the bacterial challenge but remain elevated compared with periodontally healthy subjects (Aukhil *et al*, 1988; Ebersole *et al*, 1992; Papapanou *et al*, 2004); 6) IgG antibodies indicate cumulative bacterial exposure while IgA response indicates more recent exposure (Pussinen *et al*, 2007); 7) systemic antibodies can be used to examine past exposure in edentulous subjects (Pussinen *et al*, 2003; Papapanou *et al*, 2004); 8) despite the requirement of an invasive procedure to be obtained, serum is very convenient sample that can be aliquoted and stored frozen for several years and can be used to examine immune reactivity to a vast array of oral and non-oral microorganisms; 9) because immunoglobulins are present in large amounts, minute quantities of serum can be used for their measurement; and 10) high-throughput techniques exist for the quantification of antibody response to a multitude of microorganisms at the same time (Sakellari *et al*, 1997). In fact, most of the evidence in the early studies examining the infectious hypothesis of atherogenesis used seroepidemiology to determine a correlation between exposure to cytomegalovirus, herpes simplex virus, *Chlamidia pneumoniae* and *Helicobacter pylori* and CVD (Epstein *et al*, 2009).

Infectious/pathogen burden

The large clinical trials testing if macrolid antibiotic therapy targeting *C. pneumoniae* would lead to a decrease in cardiovascular events all resulted in negative outcomes (Andraws *et al*, 2005). These studies clearly indicated that the routine use of antibiotic therapy for this pathogen was not indicated in secondary prevention of coronary events. However, these studies were often erroneously interpreted as negating the participation of infectious agents in atherosclerosis. The most important limitation of these antibiotic trials is the concept that if microorganisms contribute to atherogenesis and its complications, many such microorganisms might contribute to these biological processes (Epstein *et al*, 2009). Therefore, the infection-associated risk of atherosclerosis should correlate best with the aggregate pathogen load, what was termed 'pathogen burden' (Zhu *et al*, 2000). Prospective studies performed in coronary artery disease subjects with several years of follow-up demonstrated that the number of viral and bacterial pathogens with which an individual had been infected

predicted the incidence of acute myocardial infarction and death (Rupprecht *et al*, 2001; Zhu *et al*, 2001).

These concepts have important implications to the interpretation of the findings of studies examining the association between periodontal infections and atherosclerosis. The data discussed above outlying putative mechanisms linking oral species to atherogenesis indicated that several oral species are capable of mechanisms that might trigger the formation and progression of atherosclerotic lesions. However, most mechanistic studies have focused primarily on a limited number of periodontal pathogens. As the oral cavity can be colonized by over 500 different species, the task of examining the potential involvement of all such species is overwhelming. In addition, studies examining the exposure to oral pathogens should also take into account the potential contribution of non-oral bacterial pathogens and viruses. Several studies recognize the relevance of accounting for the exposure to non-oral pathogens and have examined the presence of molecular signatures of *C. pneumoniae* and human cytomegalovirus (HCMV) in atherosclerotic plaques, in addition to oral species (Haraszthy *et al*, 2000; Ford *et al*, 2005b, 2006; Pucar *et al*, 2007). The results from these studies varied considerably and were based on limited numbers of samples, however, a few general trends could be observed. *P. gingivalis* was the most commonly found periodontal pathogen. The prevalence of several periodontal pathogens and, particularly *P. gingivalis*, was higher than *C. pneumoniae* (Haraszthy *et al*, 2000; Ford *et al*, 2005b, 2006; Pucar *et al*, 2007), while the prevalence of HCMV was higher than any periodontal pathogen tested (Haraszthy *et al*, 2000; Pucar *et al*, 2007). Universal probes for bacteria revealed the presence of bacterial DNA that could not be accounted for by the specific probes, suggesting the presence of unidentified bacteria (Haraszthy *et al*, 2000; Pucar *et al*, 2007). These preliminary findings indicate that if an association between periodontal diseases and CVD is to be explained by the exposure to oral microorganisms, a measure of the total pathogenic burden must be determined. Hitherto, there is no consensus regarding which microorganisms should be examined and a method to assess the infectious burden (Elkind, 2010). Considering the complexity of the oral microbiota and the contribution of non-oral bacterial species and viruses to the microbial burden, obtaining a measure of the cumulative microbial burden will not be an easy task.

The oral inflammatory theory of atherogenesis

Periodontal diseases and systemic biomarkers of inflammation

The role for inflammation in the pathogenesis of atherosclerosis was established in part by examining associations between systemic biomarkers of inflammation and measurements of CVD and its consequences. Originally, it was thought that such systemic biomarkers would reflect a heightened inflammatory state at the atheromatous lesions. However, in population studies, systemic elevations of CRP and cytokine levels are not explained by the magnitude of atherosclerotic lesions detected per subject (Libby, 2002). Systemic elevations in inflammatory parameters are more associated with age, smoking and adiposity than with measures of plaque thickness. Adiposity, in particular, has been shown to explain up to 30% of the systemic inflammatory burden in population studies (Libby, 2002; Pearson *et al*, 2003). This implies that elevations in systemic cytokines can be derived not necessarily from the site of the disease (the atherosclerotic lesion), but also from other tissues such as the periodontium. These studies led to the exploration of the correlation between oral parameters of inflammation and the levels of systemic biomarkers. A recent meta-analysis of cross-sectional studies examining the association between periodontal diseases and CRP concluded that 'There is strong evidence from cross-sectional studies that plasma CRP in periodontitis is elevated compared with controls' (Paraskevas *et al*, 2008). The authors included only reports using high-sensitivity CRP measurements. The mean levels of CRP reported in 10 studies reviewed in their article were 3.41 mg l^{-1} for subjects with periodontal diseases and 1.72 mg l^{-1} for periodontally healthy individuals (Paraskevas *et al*, 2008). These data are quite relevant as an elevated risk for atherosclerosis is indicated by CRP levels $\geq$3 mg l^{-1}. Since then, recent investigations have added to the evidence that periodontal diseases are associated with elevated systemic levels of CRP (Buhlin *et al*, 2009; Gani *et al*, 2009; Nakajima *et al*, 2010). In a longitudinal study involving 11 162 Japanese men, the authors reported that for men with normal levels of CRP at baseline, the presence of periodontal disease correlated with increases in CRP levels 1 year later (OR: 1.34; 95% CI: 1.12–1.67), suggesting that periodontal diseases preceded the systemic increase in CRP (Yoshii *et al*, 2009). In addition to CRP, other studies have reported elevated systemic levels of IL-6, a major inducer of the acute phase reaction and CRP, in periodontitis subjects (Loos, 2005; Nakajima *et al*, 2010). Higher levels of fibrinogen (Kweider *et al*, 1993) and IL-18 (Buhlin *et al*, 2009) have also been reported in periodontal disease subjects. Therefore, periodontal diseases seem to be associated with a low-grade systemic inflammation, with levels of serum pro-inflammatory biomarkers being elevated in periodontitis subjects.

Sources of systemic inflammatory mediators in periodontal diseases

The increase in systemic biomarkers of inflammation associated with periodontal diseases has been interpreted as a mechanistic link between these infections and CVD. However, details of the biological processes that lead to the systemic inflammation associated with periodontal diseases are poorly understood. As discussed above, it is possible that these elevations in systemic markers of inflammation occur in response to systemic exposure to oral bacteria during bacteremias. Alternatively, it has been proposed that cytokines secreted within the periodontal tissues would find their way into the blood stream and cause a low-grade

systemic inflammation. A similar mechanism is at play in RA, where the synovial tissues are the primary site of inflammation and act as a source of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 which contributes to the 'high-grade' systemic inflammation. In support of mechanism, in RA the magnitude of the systemic inflammation is associated with an increase in several risk factors for atherosclerosis (Sattar *et al*, 2003). Furthermore, CVD and premature mortality in RA subjects correlates with the number of inflamed joints, indicating that these affected sites might release mediators of atherogenesis (Pincus *et al*, 1984). Several publications have demonstrated local elevations of pro-inflammatory biomarkers within periodontal tissues affected by gingivitis and periodontitis (Gorska *et al*, 2003; Hou *et al*, 2003; Lester *et al*, 2009). These include mediators that have been implicated in systemic inflammation such as IL-1β, IL-6, and TNF-α. However, different from RA, the contribution of these local mediators to the systemic inflammation lacks supporting evidence. In fact, periodontal inflammation has not been shown to be associated with systemic elevations of IL-1β (Mengel *et al*, 2002) or TNF-α (Meyle, 1993), although both biomarkers are abundantly present in tissue samples from periodontal disease sites both at the mRNA and protein levels (Roberts *et al*, 1997a,b; Hou *et al*, 2003), and in proximal samples of gingival crevicular fluid (Gamonal *et al*, 2000, 2003; Teles *et al*, 2010a,b). Studies examining the correlation between local (in GCF samples) and systemic (serum or plasma) levels of biomarkers in periodontitis subjects have failed to demonstrate a strong association (Gorska *et al*, 2003). Several hypotheses could explain this observation. Pro-inflammatory cytokines released in the periodontal sites might be partially consumed locally within the periodontal tissues and the excess be preferentially 'drained' by the GCF, minimizing the systemic exposure. In any case, it seems that the periodontal lesion is well circumscribed and partially isolated from the rest of the body.

An alternative hypothesis posits that subjects susceptible to periodontal diseases would present a propensity to low-grade systemic inflammations. This inflammatory phenotype would expose them to a higher risk for inflammatory diseases such as periodontal diseases and atherosclerosis, explaining their co-expression. Rheumatoid arthritis exemplifies how a pro-inflammatory phenotype might predispose subjects to CVD and possibly other inflammatory conditions. In these individuals atherosclerosis can be considered as an 'extra-articular manifestation of the syndrome' and the term 'rheumatoid vasculopathy' has been proposed (Fietta and Delsante, 2009). Similarly, a few reports have described an association between the RA and periodontal diseases (Kasser *et al*, 1997; Gleissner *et al*, 1998; Mercado *et al*, 2001), implying common inflammatory mechanisms. However, as discussed above, there is evidence that periodontal disease might precede systemic CRP elevations, suggesting that the higher levels of CRP in periodontal diseases would occur as a consequence of the disease process (Yoshii *et al*, 2009).

Effects of periodontal therapy on systemic biomarkers of inflammation

The immediate effect of tooth debridement seems to be an increase in plasma levels of TNFα, CRP, and IL-6, suggesting an acute response to the bacteremia resultant from this procedure (D'Aiuto *et al*, 2004, 2005; Ide *et al*, 2004; Tonetti *et al*, 2007). However, not all studies on the impact of bacteremias on systemic levels of cytokines have reported elevations as a consequence of bacteremic events. Forner *et al* measured the levels of six cytokines in serum samples in 20 severe periodontitis subjects 8 h after scaling (Forner *et al*, 2006b). Bacteremia was detected in 15 such subjects but only IL-6 was statistically significantly elevated, IL-8 was statistically significantly reduced, while the levels of IL-1β, TNFα, IL-10, and IL-12p70 were not affected. Furthermore, changes in serum levels of biomarkers were not associated with magnitude, duration or composition of the bacteremias. A recently published systematic review on the effects of different periodontal therapies on the systemic levels of CRP concluded that there was modest evidence that periodontal treatment was associated with reductions in CRP levels (Paraskevas *et al*, 2008). Interestingly, of the studies reviewed, baseline CRP levels were all below 2.0 mg l^{-1}, comparing to levels above 3.0 mg l^{-1} for periodontitis subjects in cross-sectional studies as previously discussed. Furthermore, reductions were rather modest: 0.4–0.6 mg l^{-1} and most studies were of short-term follow-up (maximum of 6 months). Since this review was published, a few other studies have addressed the effects of periodontal treatment on systemic biomarkers of inflammation and reported inconsistent findings. Lopez *et al* reported statistically significant increases in CRP in a group of 73 patients 6 weeks after periodontal therapy with adjunctive antibiotics from 3.6 ± 3.7 mg l^{-1} to 5.4 ± 5.7 mg l^{-1} (Lopez *et al*, 2009). By contrast, other studies reported statistically significant reductions in plasma levels of CRP, IL-6, and fibrinogen 1–3 months after periodontal therapy (Blum *et al*, 2007; Marcaccini *et al*, 2009; Vidal *et al*, 2009). Although reported decreases in CRP associated with periodontal treatment were statistically significant, they were rather modest and would not result in changes in the risk stratification for CVD. Ushida *et al* found no changes in serum CRP after three different types of periodontal therapy (Ushida *et al*, 2008).

Interestingly, a recent study examining the effects of periodontal therapy on a panel of 19 systemic biomarkers demonstrated that treatment resulted in significant mean reductions in levels of PAI-1, sE-selectin, sVCAM-1, MMP-9 and myeloperoxidase (Behle *et al*, 2009). However, only reductions in sE-selectin and sICAM and serum amyloid P were maintained 4 weeks after the end of therapy. Importantly, they found a great variability in the systemic response to periodontal treatment among studied subjects with ⅓ showing marked reductions and ¼ demonstrating significant increases in systemic biomarkers, with the remainder unchanged. The authors also reported a poor correlation between clinical, microbiological and serological

parameters of periodontal disease and changes in the inflammation biomarkers. This study illustrates how the effects of periodontal therapy on systemic biomarkers of inflammation may vary greatly among study subjects. This variability might partially explain the inconsistency in the reports discussed above.

Full-mouth tooth extraction can be interpreted as a definitive (although highly undesirable) form of treatment of periodontal diseases. Therefore, examining the effects of full-mouth extraction on systemic biomarkers associated with CVD could be quite instructive. So far, only a few publications have reported on this type of intervention to address the effects of elimination of periodontal disease on atherogenesis risk. The extraction of all remaining teeth in 10 subjects with 'end-stage periodontitis' resulted in statistically significant decreases in the mean plasma levels of CRP from 3.5 to 1.6 mg l^{-1}, 12 months after the extractions. Interestingly, this reduction occurred over several months, with most subjects only reaching values below 1 mg l^{-1}, 9 months after the intervention. Furthermore, in two subjects with very high baseline values (>6 mg l^{-1}), the extractions resulted in either no change or only a reduction to levels still above 4 mg l^{-1}, demonstrating a certain degree of variability in the response to treatment (Rahman *et al*, 2005). Another study involving 67 subjects with advanced periodontal disease, full-mouth extraction resulted in a significant decrease in plasma levels of CRP, PAI-1 and fibrinogen, which lasted up to 12 weeks after the procedure (Taylor *et al*, 2006). However, the impact of the extraction on the mean values of CRP was modest, with a mean reduction of 0.7 mg l^{-1}.

To put the magnitude of these effects in perspective, the JUPITER study, which resulted in a statistically significant reduction in the occurrence of a first major cardiovascular event ($P < 0.00001$) and death from any cause ($P < 0.02$), reported median levels of CRP of approximately 2.0 mg l^{-1} for the statin group compared to 3.5 mg l^{-1} for the placebo group during 48 months of follow-up (Ridker *et al*, 2008). These data suggest that the clinical effects associated with the use of statins on cardiovascular events required a long-term consistent reduction in CRP to levels below the high risk threshold of 3.0 mg l^{-1}. So far, no intervention study on periodontal disease subjects has been able to report this magnitude of change in systemic CRP. Periodontal therapy seems to reduce the mean levels of systemic CRP by approximately 0.5 mg l^{-1}, 1/3 of the reduction obtained with the use of rosuvastatin. Furthermore, there is little to no information on the long-term effects of periodontal therapy on the plasma levels of CRP.

Hitherto, only one multi-center study has examined the effects of therapy on the secondary prevention of cardiac events, the Periodontitis and Vascular Events (PAVE) study (Beck *et al*, 2008; Offenbacher *et al*, 2009). The study demonstrated no significant effect of periodontal therapy on serum CRP levels 6 months after treatment when data were analyzed using intent-to-treat analyses. However, the study lacked a proper control group as subjects were assigned either to 'protocol therapy' provided by the study team or to 'community care'. When subjects receiving any form of treatment were compared with subjects with no treatment in a secondary analysis, periodontal therapy resulted in a significant reduction in the proportion of subjects with CRP levels >3 mg l^{-1}. Nevertheless, this effect was only present when non-obese people were included in the analyses. The study was also compromised by the inclusion of subjects with mild periodontitis with mean ($\pm$s.d.) PD values of 2.72 mm $\pm$ 0.05 and 2.69 mm $\pm$ 0.06 for the community and protocol treatment groups, respectively.

Therefore, the intervention studies testing the effects of periodontal treatment on surrogate biomarkers of cardiovascular risk can only be considered inconclusive at this point. Even when a beneficial effect in reducing systemic CRP was reported, the magnitude of the effect might not have been sufficient to alter the cardiovascular risk of the study subjects. This observation coupled with evidence of a high degree of inter-individual variability in the systemic effects of periodontal therapy, and a lack of major changes in systemic inflammation biomarkers in response to a 'definitive therapy' such as full-mouth extraction, indicate that the impact of periodontal treatment on cardiovascular risk can only be expected to be marginal. These data are in accord with evidence that there are more important sources of systemic inflammation burden, such as adiposity and smoking and reinforce the notion that periodontal diseases confer a moderate risk to atherosclerosis and its consequences. In addition, the reduction in the risk can only be sustained for as long as reduction in systemic inflammation lasts.

Conclusion and future direction

Several periodontal pathogens and other oral species have been indisputably identified in bacteremias and in atherosclerotic lesions and several mechanisms that implicate them in the inflammatory events that lead to all stages of atherosclerosis have been proposed. Evidence also supports the notion that periodontal diseases are accompanied by low-grade systemic inflammation, as documented by an increase in plasma CRP levels. However, additional evidence linking oral bacterial exposure to this state of heightened systemic inflammation is still needed. The study of the association between the two phenomena, i.e. entry of oral bacteria into the blood stream and systemic inflammation, is hampered by the complexity of the oral microbiota and difficulties in determining a proper measure of exposure to the cumulative microbial burden. The understanding of the biological mechanisms underlying this potential association between periodontal diseases and atherosclerosis is also compromised by the existence of a series of confounding factors that might contribute to systemic inflammation and possibly affect both diseases such as age, adiposity, smoking and insulin resistance. Intervention studies have contributed little to clarify the mechanisms involved in a possible increased risk for CVDs conferred by periodontal infections. Future studies might benefit from the lessons learned from

both the PAVE and the JUPITER studies. Recruitment of a large enough sample size to compensate for covariates; inclusion of appropriate treatment groups to test the impact of the periodontal infection to the outcome measures (e.g. adjunct systemic antibiotic vs. mechanical therapy); longitudinal monitoring to examine the duration of the therapeutic effect on systemic biomarkers; and selection of subjects that present concomitantly moderate to severe periodontal disease and high systemic levels (>3.0 mg l^{-1}) of CRP might be required to clarify if periodontal therapy has a real impact on cardiovascular risk. Evidence that control of periodontal infections leads to a decrease risk for cardiovascular events, would greatly substantiate a link between both conditions.

Acknowledgements

This work was supports by NIDCR grants R01 DE017400 and U01 DE021127 to RT and R01DE16513, R01DE017684 and R01DE019412 to CYW.

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