Introduction

Periodontitis is a chronic infection that results from the interaction of periodontopathogenic bacteria and host inflammatory and immune responses and is the most common bacterial infection worldwide. Estimates reveal that 10 - 15 % of adults have advanced periodontitis, and periodontal disease can contribute to widespread oral health dysfunction and enhanced susceptibility to other systemic diseases ***(Pussinen et al., 2007).***

Bacterial biofilms are considered to be the primary etiological factor in the initiation ofgingival inflammation and the following destruction of periodontal tissues ***(Offenbacher, 1996)***. Three primary specific pathogens have been repeatedly identified as etiologic *agents namely Aggregatibacter (Actinobacillus) actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg) and Tannerella forsythia (Tf)* ***(Socransky et al., 1998).***

Although chronic exposure to bacteria and their products is a main reason for gingival inflammation and periodontal tissue destruction to occur, the major causative factor of soft- and hard- tissue breakdown associated with periodontitis is currently contributed to the host's immune-inflammatory response to bacterial challenge. Also the nature of the inflammatory response might determine the destructive character of the disease ***(Gemmell et al., 2002).***

The biologic phenotype underlying chronic periodontitis, including the biofilm and the host response, tend to vary among individuals despite a similar clinical diagnostic category ***(Offenbacher et al., 2007).*** Consequently,

disease screening should ideally be based on clinical determinations and the biologic phenotype ***(Page and Kornman, 1997).***

Other involved factors include environmental exposures, differences in genetic and also epigenetic composition ***(Page and Kornman, 1997).***

The biological changes underlying the transition process from gingival health to early inflammatory changes include local increase in vascular permeability, edema and the recruitment and activation of polymorphonuclear neutrophils (PMN) ***(Delima and Van Dyke, 2003).*** Acquired immune response becomes involved when antigen-presenting cells interact with immunocompetent cells, such as T and B lymphocytes, leading to the expansion of antibody-secreting plasma cells and the development of the chronic lesion ***(Gemmell and Seymour, 2004).***

Bacterial–host interactions at the biofilm–periodontium interface induce the synthesis of cytokines and other inflammatory mediators that induce the release of enzymes and bone-associated molecules that finally induce the alterations of the connective tissue metabolism and the destruction of the tooth supporting alveolar bone ***(Shapira et al., 2007).***

In addition to local periodontal tissue involvement, chronic infection of the periodontium together with continuous up-regulation of pro-inflammatory responses and immune mediators may assist in systemic sequel including diabetes, preterm delivery of low weight birth babies, lung inflammation, arthritis and cardiovascular diseases (CVD)**.** In fact, numerous case-control and cohort studies have explained that

periodontitis patients have increased risk for CVD, acute myocardial infarction (AMI), peripheral arterial disease and CVD, relative to patients with healthy periodontium ***(Mattila et al., 2005).***

Although the associations of periodontal diseases with CVD have been investigated in several clinical studies the pathogenic mechanisms and links between both diseases are not completely clarified ***(Buduneli et al., 2011).***

Pathogenesis of chronic periodontitis:

Development of gingival inflammation:

Accumulation of plaque at the gingival margin results in the development of gingivitis and in susceptible individuals, this will progress to periodontitis ***(Jensen et al., 1965).*** The development of gingivitis and periodontitis can be divided into a series of stages as described by ***(Page and Schroeder, 1976).***

These authors classified the development of the disease into the initial, early, established and advanced lesions.

The initial lesion occurs within the first four days following the beginning of plaque accumulation. It is a subclinical lesion that can only be observed histologically but is characterized by the formation of oedema, an increase in gingival fluid flow, an accumulation of polymorphonuclear leukocytes (PMNs) and loss of connective tissue. When plaque accumulates, bacterial enzymes and metabolic end products increase the permeability of the junctional epithelium, permitting both the ingress of further bacterial products and simultaneously the outflow of gingival fluid. This gingival fluid is importantly a serum product, which contains all the components of complement ***(Page and Schroeder, 1976).***

Activation of complement results in production of the anaphylatoxins C3a and C5a, which lead to the release of vasoactive amines from mast cells. These vasoactive substances lead to an increase in vascular permeability and the formation of oedema ***(Page and Schroeder, 1976).***

Also at this initial stage, the mast cells release preformed tumour necrosis factor-α(TNF- α), which is responsible for the expression of adhesion molecules by endothelial cells and the subsequent sticking and migration of PMNs into the gingival tissues and out into the gingival sulcus. While activation of the alternative complement pathway is essential for the vascular responses, bacterially derived chemotactic substances together with C5a are responsible for the initial migration of PMNs ***(Page and Schroeder, 1976).***

Once in the gingival sulcus however, the PMNs are unable to phagocytose the bacteria, which are now forming a biofilm and as such are firmly adherent to the tooth surface. In this situation, the PMNs release their lysosomal contents into the gingival sulcus in what has been termed ‘‘abortive phagocytosis’’. These lysosomal enzymes can then invade the tissues and contribute to the local destruction of connective tissues. At this initial stage, the lesion occupies no more than 5–10 percent of the connective tissues, and is still not clinically evident ***(Page and Schroeder, 1976).***

At approximately 4–7 days of plaque accumulation, the nature of the developing lesion turns from one consisting primarily of PMNs to one with increased numbers of lymphocytes and macrophages. This is called **the early lesion** in which vascular changes become more clear as illustrated by the activation of previously dormant capillary beds, and the development of perivascular inflammatory infiltrates ***(Seymour et al., 1983).***

As a result, there is a net increase in the flow of fluid into the affected gingival tissues, and a subsequent increase in the flow of gingival

crevicular fluid. Further concurrent widening of intercellular spaces between the epithelial cells of the junctional epithelium allows increased diffusion of bacterial products into the gingival tissues and increase of the inflammatory response. The lesion begins as small perivascular infiltrates which progressively increase in size and coalesce until they become clinically evident at around day 12 to 21 ***(Seymour et al., 1983).***

By day 21, lymphocytes make up 70 percent of the infiltrate with PMNs and plasma cells making up less than 10 percent of the total infiltrate within the tissues ***(Seymour et al., 1983).***

Increases in cell adhesion molecules such as endothelial cell leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1), together with an increase Interleukin-8 (IL-8) production by the epithelial cells, assist in establishing a fast flow of PMNs through the junctional epithelium and into the gingival sulcus, where they form a barrier against plaque microorganisms ***(Moughal et al., 1992).***

In some individuals, either due to environmental factors, their own innate susceptibility, or both, the stable lesion changes to an **established lesion** with B cell/plasma cell response with the production of high levels of Interleukin-1 (IL-1) and Interleukin-6 (IL-6) and subsequent connective tissue breakdown and loss of bone. As the connective tissue attachment to the tooth breaks down, the junctional epithelium migrates in an apical direction and a periodontal pocket forms, which becomes lined by pocket epithelium with in-growth of rete pegs into the surrounding connective tissue ***(Gemmell et al., 1997).***

Increased permeability of this pocket epithelium allows continued penetration of microbial products, the continued production of inflammatory cytokines such as IL-1, TNF-α, and Prostaglandin E_2 (PGE_2), and extension of the inflammatory process leading to continued tissue destruction ***(Gemmell et al., 1997).***

The primary identifying feature of the progressing, established lesion is the predominance of plasma cells within the periodontal connective tissues indicative of a B cell adaptive immune response ***(Seymour and Greenspan, 1979).***

The **advanced lesion** has essentially the same cellular make-up as the established lesion. The main difference lies in the overt loss of attachment that is evident clinically and histologically. It is now generally accepted that the mechanism of tissue destruction is through the effects of the immune response ***(Birkedal et al., 1993).***

Fibroblasts and macrophages are stimulated by the inflammatory cytokines IL-1, TNF-α and PGE_2 to produce matrix metalloproteinases (MMPs), which are a family of proteinases whose primary action is the degradation of the extracellular matrix. Collagen molecules are cleaved into smaller fragments, which then become denatured in the extra-cellular environment or are phagocytosed by surrounding fibroblasts. As the lesion advances, alveolar bone loss becomes evident. However, a non-infiltrated fibrous band remains adjacent to the crestal bone, which effectively encapsulates the progressing lesion ***(Nishikawa et al., 2002).***

Immunoregulation in periodontitis:

The nature of the adaptive immune response is dependent on a complex functional network between various immunological networks. T cells are central in the control of immune mediated mechanisms and in this context, the balance between the so-called Th1 and Th2 cells is crucial. T helper 1 (Th1) and T helper 2 (Th2) cells were first described by ***(Mosmann, 1986).***

Th1 cells mediate mainly cell mediated immune responses, as demonstrated by DTH, by secreting Interleukin 2 (IL-2) and Interferon gamma (INF-γ). The secondary function of Th1 cells is the suppression of B cells and plasma cells ***(Modlin and Nutman, 1993).*** In contrast, Th2 cells induce mainly B cell humoral immune responses by secreting Interleukin 4 (IL-4), Interleukin 5 (IL-5) and Interleukin 10 (IL-10) while their secondary function is the suppression of T cell mediated responses. Therefore, immunoregulatory control depends upon the balance between these two T cell subsets ***(Modlin and Nutman, 1993).***

In this concept, it is proposed that a strong innate immune response leads to the production of high levels of IL-12 by both PMNs and macrophages which lead to a Th1 response, cell mediated immunity, protective antibody and a stable periodontal lesion. In contrast, a poor innate immune response with polyclonal B cell activation leads to a Th2 response, non-protective antibody and a progressive periodontal lesion ***(Asikainen et al., 2006).***

While the Th1 /Th2 paradigm provides a possible mechanism by which periodontal lesions become progressive or remain stable, an important question that remains is, what causes some lesions to show Th1 characteristics while others show Th2 characteristics?The answers may lie in the nature of the microbial challenge as well as particular genetic and environmental susceptibility factors. Importantly, some of these factors may be clinically identifiable and modifiable ***(Kinane and Bartold, 2007).***

Nature of the microbial challenge:

There is no doubt that plaque is the main etiological agent for gingivitis and periodontitis. Over the past decade however, biofilms containing complexes including *Porphyromonas gingivalis, Tannerella forsythia, Aggregatibacter actinomycetemcomitans* and *Treponema denticola* have been related to chronic periodontitis, such that it is unlikely that a single antigen or asingle organism is responsible for the disease.Indeed, little is actually known of the biofilm specific antigens responsible for periodontal disease and of the immune response to them ***(Socransky et al., 1998).***

In ***Choi et al.(2000)*** showed that T cell clones derived from mice immunized with *P. gingivalis* alone had a Th1 profile, whereas T cell clones derived from mice immunized with *Fusobacterium nucleatum* followed by *P. gingivalis* demonstrateda Th2 profile. This may be due to the fact that *F. nucleatum* is a polyclonal B cell activator such that B cells subsequently present the *P. gingivalis* antigen ***(Choi et al., 2000).***

Further***, Gemmell et al. (1993)*** showed that if mice were immunized with *F. nucleatum,* they loss the ability to make antibody to *P. gingivalis*. This was not the case if bacteria were injected in the reverse order. These results, albeit preliminary, nevertheless show that it is possible for co-infection to modulate the immune response. The level of this modulation remains to be demonstrated but it is likely to involve the Th1/Th2 balance.

In their five-year longitudinal study, ***Cullinan et al. (2008)*** showed a direct effect between plaque complexes containing *P. gingivalis* and disease progression. No such effect was seen with complexes containing *A. actinomycetemcomitans,* nor *Prevotell intermedia*, such that these organisms were considered to be of only minor importance in periodontal disease progression.Nevertheless, it is possible that *P. gingivalis*, and hence complexes containing *P.gingivalis*, have the potential to modify the host response.

Toll-like receptors:

The discovery of toll-like receptors (TLRs) has led to a far greater understanding of innate immunity and the induction of adaptive immunity. TLRs are found on dendritic cells, neutrophils and macrophages and have the ability to recognize structures that are highly conserved across a wide variety of pathogens. Such structures include LPS, peptidoglycan, bacterial DNA, double stranded RNA and lipoprotein ***(Mahanonda and Pichyangkul, 2007).***

Given their role in innate immunity, it is likely that TLRs are important in determining the nature of the host response to plaque. TLR-2 and TLR-4, upon stimulation, may promote markedly different immune responses as determined by the resulting cytokine profiles.When stimulated, TLR-4 has been shown to promote expression of IL-12p70 and INF-γ inducible protein-10 (IP-10), which is indicative of a Th1 response ***(Mahanonda and Pichyangkul, 2007).***

Conversely, TLR-2 promotes the inhibitory IL-12p40, which is characteristic of a Th2 response ***(Strominger et al., 2001).*** These differences are reflected in differential cytokine expression by E. coli derived LPS *and P. gingivalis* derived LPS. E. coli derived LPS, which activates TLR-4induces a strong Th1 response, while *P. gingivalis* derived LPS, which activates TLR-2, induces a strong Th2 response. These findings indicate a further mechanism of susceptibility to periodontitis ***(Toshchakov et al., 2001).***

Osteoimmunology:

Extensive research in the fields of immunology and osteology has revealed an intimate relationship between inflammation/the immune system and the skeletal system ***(Takayanagi, 2007).*** The immunoskeletal interface involves centralization of the immune and skeletal functions around common cells types and cytokine effectors that control the physiological bone mass. Prolonged immune activation can lead to skeletal deterioration. This interconnection of the skeletal and the immune systems has spawned the emergence of a new field of science termed "osteoimmunology". The field of osteoimmunology offers better insights into the understanding of

pathogenesis of periodontal disease by explaining the role of inflammation in alveolar bone loss ***(Weitzmann, 2013).***

Role of Receptor Activator of Nuclear Kappa B Ligand (RANKL) in bone resorption:

Hundreds of millions of people worldwide are affected by bone-related diseases, such as osteoporosis and rheumatoid arthritis. Understanding the molecular mechanisms of bone metabolism is crucial for developing novel drugs for treating such diseases. In particular, genetic experiments showing that the receptor activator of NF -kB (RANK), its ligand RANKL, and the decoy receptor OPG are essential, central regulators of osteoclast development and osteoclast function were significant turning points in our understanding of bone diseases ***(Theill et al., 2002).***

RANKL–RANK signaling activates a variety of downstream signaling pathways required for osteoclast development. Moreover, molecular cross-talk between RANKL–RANK and other ligand–receptor systems fine tunes bone homeostasis in normal physiology and disease. Designing novel drugs that target RANKL–- RANK and their signaling pathways in osteoclasts could potentially revolutionize the treatment of many diseases associated with bone loss such as arthritis, tooth loss, cancer metastases or osteoporosis ***(Theill et al., 2002).***

Bones are constantly remodeled through the synthesis of bone matrix by osteoblasts and the resorption of bone by osteoclasts. Perturbations in inflammatory cytokines, growth factors and hormones cause an imbalance between osteoclast and osteoblast activities and can

result in skeletal abnormalities, such as osteoporosis and osteopetrosis. Osteoporosis is a devastating disease characterized by lower bone density, frequently found in older people (especially women), immobilized patients, or even astronauts as a result of experiencing zero gravity, and ultimately results in bone fractures**.** By contrast, osteopetrosis or abnormally increased bone density occurs mainly as a result of rare hereditary disorders. The discovery of the factors involved in the control of osteoclasts, and hence osteoporosis, has moved bone research into a new era ***(Karsenty and Wagner, 2002).***

These factors are the receptoractivator of NF-kB (RANK), its ligand RANKL and the decoy receptor for RANKL, osteoprotegerin. Binding of RANKL to its receptor RANK provides the crucial signal to drive osteoclast development from haematopoietic progenitor cells as well as to activate mature osteoclasts ***(Anderson et al., 1997).***

OPG negatively regulates RANKL binding to RANK and therefore inhibits bone turnover by osteoclasts. As increased osteoclast activity is observed in patients with osteoporosis, metastases or rheumatoid arthritis, the RANK–RANKL–OPGaxis appears to be the most relevant therapeutic target for all bone diseases. The next milestone after the identification of RANK and RANKL was the elucidation ofdown stream molecules that relay RANK–RANKL stimulation to osteoclast-specific developmental programs ***(Simonet et al., 1997).***

Basic characteristics of RANK, RANKL and OPG

The identification of the factors involved in osteoclast development began with OPG, which was first cloned as a potential inhibitor of osteoclastogenesis ***(Tsuda et al., 1997).*** OPG is a member of the tumor

necrosis factor (TNF) receptor superfamily, and is highly expressed in adult lung, heart, kidney, liver, spleen, thymus, prostate, ovary, small intestine, thyroid, lymph node, trachea, adrenal gland, testis, and bone marrow. Molecular binding experiments showed that OPG associates with RANKL and functions as a decoy receptor ***(Yasuda et al., 1998).***

The receptor for RANKL is RANK, a type I transmembrane protein originally cloned from dendritic cells ***(Wong et al., 1997).*** Although OPG functions as a dimer, RANK, like other members of the tumor necrosis factor receptor(TNFR) superfamily, assembles into functional trimers. RANK is ubiquitously expressed in skeletal muscle, thymus, liver, colon, small intestine, adrenal gland, osteoclast, mammary gland epithelial cells, prostate and pancreas ***(Fata et al., 2000).*** Four groups independently cloned RANKL as an apoptosis regulatory gene, a ligand for RANK, and a binding partner of OPG ***(Boyle et al., 2003).***

Although high RANKL expression can be found in lymph nodes, thymus and lung, only low levels of RANKL can be detected in spleen, bone marrow, peripheral blood, leukocytes, heart, placenta, skeletal muscle, stomach or the thyroid ***(Fata et al., 2000).***

The roles of RANK, RANKL and OPG in vivo

One of the essential, albeit not exclusive, roles of RANK and RANKL in vivo is the regulation of bone turnover via osteoclasts. Recombinant RANKL and activating antibodies against the RANK extracellular domain can stimulate RANK and, hence, promote osteoclastogenesis ***(Hsu et al., 1999).***

Mice with a disruption in either RANKL or RANK show severe osteopetrosis and defective tooth eruption resulting from a complete lack of osteoclasts. These genetic experiments proved that RANKand RANKL are essential for osteoclastogenesis ***(Li et al., 2000).*** By contrast, mice lacking OPG, the decoy receptor for RANKL, show osteoporosis resulting from increased numbers and activities of osteoclasts ***(Mizuno et al., 1998).*** In line with mouse models, mutations in RANK and OPG have been identified in patients that have bone disorders. For instance, two heterozygous insertion mutations were found in exon 1 of RANK in affected members of four families with familial osteolysis or familial Paget disease. One mutation was a duplication of 18 bases and the other was a duplication of 27 bases, and both affected the signal peptide region of the RANK molecule, as well as causing an increase in RANK-mediated NF-kB signaling, resulting in increased osteolysis and bone remodeling ***(Hughes et al., 2000).***

In addition to the local regulation of osteoclasts, one surprising finding was that RANKL is upregulated on T cells upon activation ***(Horwood et al., 1999).*** It has, therefore, been speculated that RANK–RANKL might be an important regulator of interactions between T and dendritic cells and might have a role in the regulation of the T-cell-dependent immune response. However, dendritic cells, where RANK was initially identified, appear normal in their function to induce T cell proliferation ***(Kong et al., 1999).***

Moreover, RANK stimulation is not essential for experimental autoimmune myocarditis in mice**.** Intriguingly, RANKL deficient mice lack all lymph nodes, and the administration of RANK-Fc (a fusion