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Peripheral blood monocyte responses in periodontitis

Abstract: Periodontitis results from the interaction of bacteria on the tooth surfaces and the host immune response. Although periodontal pathogens are essential for the initiation and progression of the disease, the tissue damage in periodontitis is primarily mediated by the host immune response. Differences in the susceptibility to the disease and in the clinal outcome of the therapy seem to be less dependent on genetics but more on lifestyle factors, like smoking, overweight, stress and nutrition. It has been shown that these lifestyle factors may modulate the immune response and therefore influence the initiation and progression of the disease. To study the host immune response, whole blood cell cultures (WBCC) stimulated with lipopolysaccharide (LPS) have been widely used and they specifically reflect the behaviour of monocytes. It has been shown that peripheral blood monocytes in LPS-stimulated WBCC from non-smoking periodontitis patients display a T-helper 2 (Th2)-promoting phenotype in comparison with controls. After periodontal therapy, this phenotype reversed and was comparable with controls. However, in smoking but treated patients, the Th2-promoting phenotype of monocytes still remained. Therefore, the aberrant phenotype of monocytes in the peripheral blood from periodontitis patients is likely to be a systemic response to exogenous and endogenous danger molecules released or induced by the periodontal infection or by smoking. It can be concluded that periodontal therapy in non-smoking periodontitis patients has beneficial health effects and that smoking cessation should be an integral part of the therapy as well for general health reasons as for the clinical outcome.

Key words: IL-12; IL-8; macrophage chemoattractant protein-1; periodontal therapy; periodontitis; peripheral blood monocytes; PGE_2; smoking; T-helper 2 cells; whole blood cell cultures

Introduction

Periodontitis is a chronic inflammatory and destructive disease of the teeth-supporting tissues, that is, connective tissue from the periodontal ligament and alveolar bone. This inflammatory condition will, if left untreated, eventually lead to loosened teeth and subsequent exfoliation. Clinically, the disease is characterized by deep probing pocket depths as a result of connective tissue attachment loss and bleeding upon probing owing to the inflammation. Periodontitis results from the interaction with environmental factors, that is, mainly Gram-negative bacteria or their cell wall components like lipopolysaccharides (LPS), accumulating on the tooth surfaces and the host immune response (1).

Dates:
Accepted 10 June 2012

To cite this article:
Int J Dent Hygiene **10**, 2012; 229–235
DOI: 10.1111/j.1601-5037.2012.00572.x
Fokkema SJ. Peripheral blood monocyte responses in periodontitis.

Although the inflammatory and immune responses within the periodontal tissues resemble those seen elsewhere in the body, there are significant differences attributable to the unique anatomical features of the periodontium, that is, the passage of the tooth through the soft-tissue integument into the oral cavity and its solid non-shedding surface. This inevitable feature of the tooth surface leads to undisturbed bacterial colonization when daily hygiene regimens are omitted and so-called biofilms develop, that is, bacterial communities that are in a continual state of flux. Nowadays, more than 12 000 different bacterial species have been indentified in the oral cavity by culture-independent molecular methods such as pyrosequencing (2). However, the role of these multitude of microorganisms in the formation of the biofilm and the localization within the biofilm is still largely unknown.

Periodontal disease has been referred to as a polymicrobial infection which contrasts infectious diseases in the classical way, that is, a susceptible individual is exposed to a single pathogen, which colonizes the host and causes the signs and symptoms of the disease via the production of specific virulence factors. Despite the large diversity of the microorganisms within the subgingival biofilms, only a small number have been associated with disease. In this respect, *Aggregatibacter actinomycetemmcomitans* is believed to be a risk factor for periodontitis and therefore aetiologically important (3, 4), while *Porphyromonas gingivalis* and *Tannerella forsythia* are associated with disease progression (5, 6). Although subgingival periodontal pathogens are essential for the initiation and progression of the disease, it is the resulting host immune response that primarily mediates the tissue damage in periodontal disease (7, 8). Therefore, the outcome of periodontal disease is determined by the effectiveness of an individual's immune response towards the microbial environment and which is regarded as the susceptibility to the disease.

Susceptibility

That not all individuals are equally susceptible to periodontal disease has been shown by longitudinal human studies on the natural history of periodontal disease, performed in rural populations deprived from any dental care. These studies showed that despite the high prevalence of (major) periodontal pathogens, only a small proportion of the population (8%) developed a generalized severe form of periodontitis leading to the loss of all teeth at the age of 45 years (9, 10). More then 80% of the population developed a moderate type of periodontitis that gradually deteriorated during life. Also studies in industrialized countries revealed that a small portion of the population is on a high risk of developing severe periodontitis (11, 12). Even studies evaluating the long-term effect of periodontal therapy in patients receiving regular periodontal care showed that a small subpopulation of the treated patients exhibited progressive disease leading to the loss of almost all teeth, irrespective of the type of therapy applied (13, 14).

The fact that not every individual is equally susceptible to periodontal disease and not every individual is responding favourably to periodontal therapy may suggest differences in genetic backgrounds between susceptible and non-susceptible individuals. In this respect, probably the most powerful method to study genetic aspects of periodontal disease is the twin model. Studying phenotypic characteristics of twins is a method of differentiating variations owing to environmental and genetic factors. Especially, when monozygous twins, who are separated at birth and reunited in adulthood, are studied. In this way, the effect of shared genes can be examined without the confounding effects of a common environment. Such twin studies have suggested a substantial role for genetic factors in the aetiology of periodontitis (15, 16). However, the main limitation of these studies was the selection of subjects based on the twinship rather than the periodontal condition, resulting in populations with mild periodontal breakdown. To overcome this limitation, a twin study was recently conducted by selecting the twin pairs on the basis of one sib of a twin pair having moderate-to-severe periodontitis (17). The results showed a discordance in loss of attachment and alveolar bone for monozygotic as well as dizygotic twins, and therefore, it was concluded that the magnitude of the genetic effects on disease severity has previously been overestimated. In this respect, the role of epigenetics should be mentioned in understanding the origins of complex diseases like periodontitis and the susceptibility to the disease (18). Epigenetics is engaged with heritable and reversible modifications in the gene function by diet, pollution, infections and lifestyle factors effecting cell functioning. This indicates that during life, variation in gene function occurs in response to environmental factors and is independent of the basic DNA code.

As periodontal disease is a complex or multifactorial disease and the susceptibility to advanced forms of adult periodontitis seems not to be restricted to genetics, it is recognized that in addition to environmental factors, lifestyle factors also play a pivotal role in the pathogenesis of the disease. In this respect, smoking is recognized as an important risk predictor in periodontitis, that is, smokers have more severe forms of periodontitis, in general respond less favourable to periodontal (non)-surgical therapy (19–21) and continue to be infected with periodontal pathogens after periodontal therapy (22). It is well acknowledged that cigarette smoking affects the oral environment and ecology as well as the gingival tissues, the vasculature, the inflammatory response and the homoeostasis and healing potential of the periodontal connective tissues (23). In addition, smoking may affect the innate immune responses by shifting the net balance of neutrophil activities into more destructive directions. Therefore, it is evident that smoking affects the host immune response, which provides mechanisms for the increased susceptibility to periodontitis and the poorer response to treatment.

Other lifestyle factors that may promote the progression of periodontitis are overweight, physical activity, stress and nutrition. A large epidemiological study among non-diabetic, non-smoking adults has shown a significant relationship between body weight and periodontitis (24). In addition, the positive

relationship between chronic periodontal disease and obesity has recently been confirmed in a systematic review (25). It has been suggested that products of oxidative damage and advanced glycation end products, associated with metabolic syndrome, might promote periodontal disease. On the other hand, periodontitis itself could be a source of oxidative stress and in turn accelerate the onset of insulin resistance and metabolic syndrome. As overweight is an imbalance between energy consumption and intake, low physical activity and a poor diet have also been associated with increased risks of periodontitis (26, 27). Stressful life events and negative emotions modulate the immune system, and the association between stress and disease is particularly strong for infections, inflammation and wound healing (28). In particular, coping behaviour and personality traits seem to be important in relation to the increased risk of severe forms of periodontitis (29). Finally, nutrition is believed to be of great importance in the susceptibility to periodontitis, as it has been implicated in a number of inflammatory diseases and conditions, including cardiovascular diseases, type 2 diabetes, rheumatoid arthritis and inflammatory bowel disease, all of which have been associated with periodontitis (30). As to date, the literature has shown that periodontal disease is associated with low serum/plasma levels of various micronutrients, principally vitamin D, vitamin C, and other antioxidants. Moreover, these micronutrients not only play an important role in various physiological but also pathogenic cell processes, such as immune responses and oxidative stress.

Taken together, the fact that not every individual is equally susceptible to periodontal disease and not every individual is responding favourably to periodontal therapy is more likely to be associated with lifestyle factors then genetics and the host immunoinflammatory response seems to be of fundamental importance.

Host immune responses

In response to an antigen, the host reacts with a non-specific innate immune response and a subsequent specific adaptive immune response. The innate immune response is the first line of defence and acts through the recruitment of mainly phagocytic cells, such as neutrophils and macrophages, the activation of the complement system and the identification of foreign substances by antigen-presenting cells. During the early events in the immune response, cytokines are produced by the tissue cells causing a gradient across the endothelium and facilitating the transmigration of peripheral blood neutrophils and monocytes. Crossing the endothelial tissues, monocytes start to differentiate into either macrophages, important scavengers in the inflamed periodontal lesion, or immature dendritic cells (DCs), highly specialized antigen-presenting cells. Subsequent maturation of DCs in the periodontal lesion occurs upon activation by microbe-associated molecular patterns and by inflammatory mediators, that is, cytokines, derived from the local microenvironment (31, 32). The matured DCs carry this information to the draining lymph nodes and instruct the cells of the specific immunity by eliciting different classes of T-helper cell responses.

Three types of T-helper (Th) cells develop from the same T-cell precursor: Th type 1 (Th1), Th type 2 (Th2), Th type 17 (Th17) cells that direct cellular, humoral and innate immune responses, respectively (33, 34). In addition, naïve T cells may also differentiate into regulatory T cells (Treg), which dampen down the immune response and prevent autoimmunity (35). Within the lymph nodes DCs present pathogen-derived peptides to specific T-cell receptors in the context of class II major histocompatibility complex, determining the specificity of the response, provide T cells with co-stimulation through CD28, determining whether or not an immune response will occur and how strong this response will be and release cytokines, driving the polarization of non-committed Th cells into either Th1, Th2 or Th17 cells. In this respect, interleukin (IL)-12 is an important and crucial factor, directing the development of Th1 cells and maintaining such responses (36). Alternatively, Th2 polarization may be induced by prostaglandin (PG)E_2 either directly (37, 38) or indirectly through the inhibition of IL-12 production by DCs (39, 40). Whereas IL-1, particularly IL-1α, seems to drive the Th17 differentiation in humans (41). It has become clear that cytokines play a major role in the regulation of T-cell subsets and interact and function in networks (42). Therefore, T cells are considered to exhibit 'functional plasticity', and this phenomenon is influenced by the cytokine milieu (43). Furthermore, the Th subsets Th1, Th2 and Th17 are characterized by their different profiles of regulatory cytokines. Th1 cells produce interferon (IFN)-γ, whereas Th2 cells mainly produce IL-4 and IL-13 (33). The Th17 cells produce in addition to IL-17 also IL-22 and IL-26 (31) and Treg cells secrete transforming growth factor-β (TGF-β) and IL-10 (35).

It is generally accepted that the stable lesion in periodontitis is largely mediated by cells with a Th1 cytokine profile, while the progressive unstable lesion involves Th2-like cells leading to increased B cells and plasma cells (44, 45). The discovery of the new Th17 subset has extended the Th1/Th2 paradigm and leads to a deeper understanding of host–pathogen interactions in the periodontium (46). The immunoregulatory control of Th1/Th2/Th17 cytokine profiles seems fundamental in determining the pathophysiology of periodontitis; however, the development of such specific Th subsets is dependent upon the presentation of antigens by the antigen-presenting cells, that is, DCs, and therefore, DCs play a crucial role in the control of the immunity by orchestrating the immune response.

LPS-stimulated WBCC reflect the behaviour of monocytes

To study host immune responses, whole blood cell cultures (WBCC) stimulated with non-specific microbe-associated molecular patterns, such as LPS, have been widely used. In response to LPS, CD14 is required together with the accessory protein MD-2 and the LPS-binding protein. CD14 is the principal receptor for LPS on myeloid cells that lack, however, a

cytoplasmic domain and therefore presents LPS to Toll-like receptors (TLRs). TLRs are pattern recognition receptors essential for the cellular response to bacterial cell wall components, and in mammals, there are at least 10 members of the TLR family. Upon LPS stimulation and the subsequent activation of TLRs, intracellular signalling leads to the synthesis of inflammatory mediators, such as cytokines, chemokines and prostaglandins (47).

In general, it has been demonstrated that CD14 and TLR-4 are highly expressed by peripheral blood monocytes and that the monocyte is the key orchestrator of LPS responsiveness in WBCC (48). Thus, studies and experiments revealed that WBCC stimulated with LPS reflect the behaviour of monocytes in particular as has been shown in parallel cultures of whole blood and freshly isolated monocytes as well as in kinetics (39, 49–53). In addition, it was shown that the purified monocytes but not CD14-depleted peripheral blood mononuclear cells or granulocytes were responsible for the production of cytokines following stimulation with LPS (39, 53). Furthermore, the reflected performance of monocytes in LPS-stimulated WBCC was found to occur at relatively low levels of LPS, as cytokine production by neutrophils required much higher amounts of LPS (52). Therefore, WBCC as a model has been applied to study aspects of the pathophysiology of periodontitis.

Whole blood cell cultures have the disadvantage of the complexity being a mixture of cells and plasma, but these cultures also have some advantages over isolation procedures in the *ex vivo* production of cytokines. Owing to minimal handling of cells in the WBCC, the likelihood of endotoxin contamination and cellular activation is reduced. In addition, the integrity of the cellular interactions is maintained as best possible. Therefore, the WBCC system may represent more closely the natural environment and seems suited for studies into periodontitis, which is now further discussed below.

Monocyte response in periodontal health and disease

Studies employing WBCC showed no difference between non-smoking periodontitis patients and non-smoking control subjects in the release of major pro- and anti-inflammatory cytokines, that is, tumour necrosis factor (TNF)-α, IL-6 and IL-10, indicating that there is not a general failure of monocytes in periodontitis patients to produce cytokines (54). In the LPS-stimulated WBCC, the release of chemokines was also analysed, showing higher levels of IL-8 and a trend towards enhanced macrophage chemoattractant protein (MCP)-1 levels in the cultures of the non-smoking periodontitis patients in comparison with control subjects (55). As IL-8 and MCP-1 are belonging to the first line of defence molecules, recruiting neutrophils and monocytes, respectively, to the site of inflammation, their higher expression in periodontitis patients may reflect an activated state of the immune system. Furthermore, the release of the pro-inflammatory cytokine IL-1β was somewhat lower in patients (54), suggesting a depressed cellular immunity in periodontitis patients. It has been shown that IL-1β participates in the priming of human Th lymphocytes and co-stimulates Th cell responses by up-regulating the IFN-γ production (56, 57). In accordance with isolated monocytes (58), WBCC from periodontitis patients released on average 2–3 times higher levels of PGE_2 compared with control subjects. WBCC from patients released lower levels of IL-12p70 (the biologically active heterodimer of IL-12), while the levels of IL-12p40 did not differ (54). PGE_2 is known to exert a Th2-promoting activity either directly by inhibiting the Th cell production of IFN-γ (37, 38) or via an IL-12-antagonistic way by suppressing the production of IL-12p70 (39, 40), while IL-12p70 is obligatory for the induction of Th1 cell responses (36). Therefore, based on the WBCC system, it was shown that peripheral blood monocytes from non-smoking periodontitis patients have a Th2-promoting phenotype. Consistent with this observed Th2-promoting phenotype of the non-smoking peripheral blood monocytes is the depressed Th1-cell response in periodontitis patients (59–62). These reported depressed cellular immune responses in the peripheral blood mononuclear cells from periodontitis patients are most likely not a defect in the T cells, but are attributable to elevated levels of PGE_2 also released by the monocytes in the WBCC. Interestingly, the Th2-promoting phenotype of monocytes and the consequently decreased Th1-cell response have also been found for other chronic inflammatory diseases, like atopic dermatitis, allergic asthma and rheumatoid arthritis, and for infectious diseases, like HIV-infection (49, 53, 63–66).

Monocyte response after periodontal therapy

The bias of peripheral blood monocytes from periodontitis patients to promote Th2-cell responses, and the consequently induced aberrant function of the peripheral blood lymphocytes towards decreased Th1-responses, might be an intrinsic or acquired characteristic of the monocytes. To explore whether this bias of the monocytes from periodontitis patients is derived from the chronic periodontal inflammation, periodontal treatment studies should shed light on this issue. Previous studies showed that following non-surgical periodontal therapy, the depressed autologous mixed lymphocyte response returned to normal limits in periodontitis patients (61, 67). The Th2-promoting phenotype of the monocytes from periodontitis patients, as evaluated in LPS-stimulated WBCC, also reversed after extensive non-surgical periodontal therapy including the use of systemic antibiotics (68). The levels of IL-12p70 increased strongly 3 months after periodontal therapy, whereas the MCP-1 levels decreased and the PGE_2 levels showed a trend towards reduction. In addition, on average the ratio of the PGE_2 and IL-12p70 concentrations decreased two times. As has been shown in the literature, the net IFN-γ production of T cells is largely determined by the ratio of DC-derived PGE_2 and IL-12p70 at the time of T-cell activation (69). The decreased PGE_2 to IL-12p70 ratio indicates that the peripheral blood monocytes from patients will favour the promotion of Th1-cell responses instead of Th2 responses after periodontal

therapy. Therefore it can be concluded that the cytokine profile as evaluated in the WBCC is an acquired characteristic.

After periodontal therapy, also changes in the MCP-1 and IL-12p70 levels consistently correlated in a reversed way, that is, decreased MCP-1 levels and increased IL-12p70 levels. Moreover, two out of eight treated patients that showed a less favourable treatment result, that is, having either more residual bleeding upon probing or more deeper probing depths, showed neither a decrease in the levels of MCP-1 nor a strong increase in the IL-12p70 levels. This correlation between MCP-1 and IL-12p70 suggested a possible functional relationship between both cytokines. Indeed in mice it was shown that MCP-1 had a suppressive effect on IL-12. The treatment of mice with MCP-1 resulted in a reduced production of IL-12p70 within mucosal tissues (70). In addition, in a mouse model of endotoxic shock, the exogenous administration of MCP-1 significantly protected mice from endotoxin-induced lethality (71). The protection correlated with low IL-12p70 levels in serum and resembled the protection observed after anti-IL-12p70 treatment. Moreover, MCP-1 has been shown to suppress the production of IL-12p70 in activated human monocytes, while other chemokines seemed to have no or little effect (72).

As stated before, lifestyle factors also play an important role in periodontitis and therefore the effect of smoking on the *ex vivo* production of cytokines by the monocytes and the subsequent lymphocytic response in peripheral blood have recently been investigated. It was shown that the monocytes in the WBCC of periodontal maintenance patients that smoke on average 14 cigarettes a day for 35 years also display a Th2-promoting phenotype in comparison with non-smoking maintenance patients, as reflected in a lower IL-12/IL-10 ratio and lower IL-1β levels for smokers (73). In addition, it was shown that smoking was associated with a Th2 phenotype, that is, stimulated T cells in the WBCC released elevated levels of IL-13 (74). Interestingly, this Th2 phenotype of the peripheral blood T cells was independent of the fact whether the patient was treated, and was also found in smokers without destructive periodontal disease. These data suggest that smoking induces a Th2 profile in the peripheral blood cells regardless the disease state, potentially increasing the risk of periodontal breakdown, while periodontal therapy in non-smoking patients reverses the Th2-(promoting) phenotype of the peripheral blood cells to a periodontally stable Th1 phenotype.

The aberrant phenotype of the peripheral blood monocytes, as seen in patients suffering from destructive periodontal disease but also from other chronic inflammatory diseases, has been extrapolated to the instructive nature of the DCs within the peripheral tissues. However, the final phenotype of maturated DCs within the periodontal tissues is determined by pathogens or their products and cytokines derived from that local tissue microenvironment, that is, epithelial cells and fibroblasts (48, 49). Owing to the high functional flexibility of the immature DCs in response to environmental factors, the definite promotion of the adaptive immunity is decided within the peripheral tissues themselves. As the similar plasticity has been observed for peripheral blood monocytes as for monocyte-derived DCs, it is most likely that the immature blood-derived precursor cells respond in a functionally flexible way towards changes in their local microenvironment, that is, the peripheral blood. Therefore, the reactivity of peripheral blood monocytes should be interpreted as a systemic response to exogenous and endogenous danger molecules released or induced by the periodontal infection rather then a Th2-promoting and therefore disease progressive phenotype within the periodontal tissues.

Conclusion

It can be concluded that peripheral blood monocytes are highly responsive cells, that is, produce high levels of cytokines in response to relatively low concentrations of LPS. In addition, peripheral blood moncytes possess a functional plasticity, that is, during disease the levels of the Th2-promoting cytokine IL-12 are reduced and reversed after periodontal therapy, and comparable to the levels in controls. Moreover, the Th2-promoting capacity of the peripheral blood monocytes is in accordance with the Th2 profile of the T cells in the peripheral blood, that is, enhanced levels of IL-13. Furthermore, the peripheral blood monocytes adapt their function to the conditions they are encountered in, for example, the circulation. Changes in this environment, induced either by systemic responses or by danger molecules released from the diseased tissues or by toxic molecules from smoking, result in a different functional profile of the peripheral blood monocytes. Therefore, it is most likely that the aberrant phenotype of the peripheral blood monocytes from periodontitis patients is a result of the chronic inflammatory and immunological response in the periodontal tissues, that is, transmigration of bacteria and LPS into the circulation and formation of systemic immune complexes, causing phenotypic changes in monocytes. In addition, periodontal therapy reverses the aberrant phenotype of the peripheral blood monocytes which may have positive general health effects. However, this beneficial effect is not found in smokers, as the aberrant phenotype of the peripheral blood cells maintains despite periodontal therapy. This may explain the less favourable response to periodontal therapy in smokers and the increased risk of cardiovascular diseases in smokers. Therefore smoking cessation should be an integral part of periodontal therapy, not only for better clinical outcomes of the therapy but also for general health reasons.

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