Susceptibility to periodontitis. Studies with LPS-stimulated whole blood cell cultures
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CHAPTER 6

The Possible Role of Plasma IL-8 and MCP-1 Levels in the Relationship between Periodontitis and Atherosclerosis

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ABSTRACT

It is believed that the periodontal infection may contribute to the development and progression of atherosclerosis by eliciting systemic responses. The present study investigated whether periodontitis results in systemically elevated levels of pro-inflammatory mediators (interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, IL-8, monocyte chemoattractant protein (MCP)-1 and C5a), and the effect of therapy on these levels. Peripheral blood samples were taken from 19 non-smoking periodontitis patients and 19 gender-matched controls. Blood samples were taken again 3 months after therapy from 9 out of the 19 patients undergoing non-surgical periodontal therapy (n=8) or full mouth tooth extraction (n=1). Plasma IL-8 levels were not different between untreated patients and controls, but decreased after therapy. The plasma MCP-1 levels were higher in patients than controls, and did not change after therapy. More patients than controls (8 versus 1) displayed elevated plasma levels of the complement product C5a, although no differences existed between the mean levels in controls and patients before and after therapy. The plasma levels of IL-1β and TNF-α were only measurable in 2 patients and 2 controls. For IL-6 the prevalence of sero-positivity revealed no differences between groups. In conclusion, after therapy the IL-8 levels in plasma decrease, most likely since circulating monocytes are no longer activated. The MCP-1 levels do not change after therapy and continue to be higher in periodontitis patients, suggesting that periodontitis may has contributed to a vascular response that is not resolved by periodontal therapy.

INTRODUCTION

Human cardiovascular disease has been associated with a history of infections with gram-negative bacteria, including Helicobacter pylori and Chlamydia pneumoniae, as well as cytomegaloviruses (CMV) (1). In addition, associations of cardiovascular
disease and periodontitis have also been reported (2). The major cause for cardiovascular disease is atherosclerosis, a chronic inflammatory disease of the blood vessel walls, believed to be induced by endothelial dysfunction or injury (3). An important inducer of this endothelial injury is oxidized low-density lipoprotein that is deposited in plaques on the arterial wall, and that subsequently plays a role in the increased adherence of monocytes and their migration into the vascular wall (4, 5). It has been shown that infections of endothelial cells with agents such as CMV or C. pneumoniae, but also systemic inoculation with endotoxin and inflammatory cytokines, cause endothelial cell dysfunction and activation, inducing cell lysis, increased leukocyte adhesion and pro-coagulant activity (6-8). In the same way periodontal infection could contribute to the development and progression of atherosclerosis (9).

The potential role of periodontitis in the chronic vascular inflammation has been shown by findings that periodontal pathogens may be present in atherosclerotic plaques (10), and that systemic inoculations with P. gingivalis accelerate the progression of atherosclerosis in a special murine model (11). In addition, it has been shown that periodontitis induces systemic release of endotoxins (12), and elicits a systemic acute phase reaction, including the activation of complement and the induction of acute-phase proteins (13). It has been shown that the expression of C-reactive protein (CRP) and fibrinogen are enhanced in periodontitis (14-17). The production of such acute-phase proteins is regulated by the early-response cytokines interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6 and IL-8, which are released by alarmed monocytes or tissue macrophages (18,19). In periodontal disease the prevalence of sero-positivity for IL-6 was found to be higher (16); IL-6 probably provokes the augmented expression of CRP in the liver (20).

If the periodontal infection is not treated and pursues, it will result in a continuous chemo-attraction of circulating leukocytes to the chronic inflamed periodontal tissues. Major chemo-attractants in this respect are IL-8, MCP-1 and C5a. The chemokines IL-8 and MCP-1 also play important roles in the early events of atherosclerosis. Both
causing rapid monocyte adherence onto vascular endothelium, and in addition, MCP-1 also induces transmigration and activation of monocytes into the intima (21, 22). Moreover, mice lacking the receptor for MCP-1 or being deficient for MCP-1 have decreased atherosclerosis, while mice over-expressing MCP-1 show accelerated atherosclerosis, indicating a crucial role for MCP-1 in the pathogenesis of atherosclerosis (23-25).

Therefore, the present study investigated plasma levels of IL-1β, TNF-α, IL-6, IL-8, MCP-1 and the complement component C5a in untreated patients suffering from periodontitis, in control subjects without destructive periodontal disease, and in patients following periodontal therapy, including a full mouth tooth extraction in one case.

MATERIALS AND METHODS

Study population
Nineteen untreated periodontitis patients (mean age 37 years; range 26-45 years; 6 non-Caucasians), who were referred to the Department of Periodontology of the Academic Center for Dentistry Amsterdam (ACTA), were selected on the following criteria: presence of periodontal bone loss > ⅓ of the root length at ≥ 2 teeth per quadrant of the mouth as visible on dental radiographs. In addition to that, only non-smoking patients were selected, i.e. those who had never smoked or had ceased smoking more than 10 years ago. A control group of 19 non-smoking subjects was selected on the basis of absence of bone loss on dental radiographs. The control subjects (mean age 37.5 years; range 27-45 years; 5 non-Caucasians) were recruited from patients of the ACTA referred for the treatment of dental caries. All participants were free from systemic diseases and usage of medication, and reported no clinical symptoms of bacterial, viral or parasitic infections at the time of the study.
Approval by the Institutional Review Board on human studies was obtained and all subjects signed an informed consent.

Eight out of the 19 periodontitis patients (mean age 34 years; range 26-42 years; 4 non-Caucasians) volunteered to participate in a specifically designed treatment protocol. The study treatment protocol consisted of oral hygiene instructions, full mouth scaling and rootplaning within 24 hours supported by systemic antibiotics and 3 months rinsing with chlorhexidine twice daily (Chapter 3). Following periodontal therapy, the mean percentage of shallow probing depths (≤ 4 mm) increased from 40 to 90%, and the percentage of deep probing depths (≥ 5 mm) decreased reciprocally to 10%. On average the bleeding on probing scores decreased from 80% to 20% after therapy. Additionally, 1 patient, a 43-year-old Caucasian male with generalized terminal adult periodontitis, was treated by full mouth tooth extraction. Due to the hopeless condition of all teeth, periodontal therapy was impossible.

**Blood sampling**

Peripheral blood samples were obtained from all untreated patients and control subjects. In addition, 3 months after periodontal and full mouth tooth extraction therapy blood was obtained again from the 9 treated patients. Before each blood collection the body temperature was measured, in order to exclude systemic infection. Non-fasting venous blood was collected in an EDTA(K3) containing tube (Becton Dickinson Vacutainer System Europe, Meylan, France). The tube was immediately put on ice and plasma was prepared within 2 hours. The aliquots of plasma were stored at -80°C until determination. Specifically, EDTA plasma samples were used for the analysis, since coagulation induces complement activation *in vitro* as well as the production of IL-8 in monocytes (26, 27).

**Biochemical analyses**

The plasma levels of the cytokines IL-1β, TNF-α and IL-6, and of the chemokine IL-8 were measured using commercially available ELISAs (PeliKine Compact™ human
ELISA kits, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, NL) according to the manufacturers instructions. A specific solid-phase sandwich ELISA also measured the levels of the chemokine MCP-1 in the plasma samples. The used specific monoclonal antibodies, neutralizing antibodies and the recombinant human MCP-1 were purchased from R&D Systems (Abington, UK). The bounded cytokines and chemokines were detected using streptavidin-poly horseradish peroxidase conjugate (CLB, Amsterdam, the Netherlands) and tetramethylbenzidine/hydrogen peroxide was used as a substrate (Biosource Europe SA, Nivelles, Belgium). Interference of plasma components in the ELISAs was prevented by the use of a high performance ELISA buffer developed at the CLB (Amsterdam, NL). The natural chemo-attractant C5a was quantified using a sandwich ELISA based on a monoclonal antibody to a neoepitope on C5a/C5a desArg, as previously described (28). The major advantages of this method are the superior sensitivity compared to commercially available radioimmunoassays for C5a desArg and the lack of cross reactivity of the Mabs employed to native complement component C5 (29). From the assays, measurable values were obtained above 4.5 pg/ml for IL-1β, 4 pg/ml for TNF-α, 2 pg/ml for IL-6 and IL-8, 10 pg/ml for MCP-1, and 20 pg/ml for C5a. Below these levels positive signals were interpreted as sero-positivity. The C5a levels in plasma were analyzed in the following ways. First, individual values per group were compared and secondly, the number of subjects with elevated C5a levels was explored. Elevated levels of C5a were defined as levels above 30 ng/ml (29, 30).

Statistical analysis
SPSS package (version 10.0 for Windows, Chicago, IL, USA) was used for data analysis. Data analyses on the patient and control group were performed by means of an analysis of covariance, whereby the age and race of the study subjects were used as covariates. Comparisons between the plasma levels before and after therapy were performed by a paired Wilcoxon. The prevalence of sero-positivity and
elevated C5a levels were analyzed by a Fisher's exact test. P-values < 0.05 were considered significant.

RESULTS

The plasma samples of all subjects showed measurable levels of IL-8. The mean levels of the untreated patients (6.1 ± 2.0 pg/ml) did not differ significantly from the control subjects (5.3 ± 1.4 pg/ml), although there were some periodontitis patients showing higher IL-8 values in comparison to controls (Figure 1A). After periodontal therapy, including the case with full mouth tooth extraction, the plasma IL-8 levels decreased in the patients (Table 1). Moreover, the treated patients tended to have even lower IL-8 levels compared to the levels of the control subjects (mean values 4.4 versus 5.3 pg/ml, \( P = 0.06 \)).

The plasma MCP-1 levels in untreated periodontitis patients were significantly higher compared to the levels in control subjects (Figure 1B). Several patients displayed plasma MCP-1 levels that were nearly 2-fold higher than the mean plasma levels seen in controls. Three months after therapy, the MCP-1 levels in plasma were definitely not changed, as can be seen in Table 1.

The plasma levels of the complement component C5a showed a wider range for the patients than for the control subjects (Figure 1C and Table 1). When the plasma C5a levels of the untreated patient group (24 ± 13 ng/ml) were compared with those of the control subjects (19 ± 7 ng/ml), there was no statistical difference. However, the prevalence of untreated patients displaying elevated levels of C5a (> 30 ng/ml) proved to be higher (8 out of 19) than the number of controls (1 out of 19) (\( P = 0.02 \)). Three months after non-surgical periodontal therapy or full mouth tooth extraction, the mean plasma levels of C5a were not changed (Table 1).

The plasma levels of the immediate early-response cytokines IL-1β and TNF-α were
Fig. 1. The individual plasma levels of IL-8 (A), MCP-1 (B) and C5a (C) measured in 19 untreated periodontitis patients and 19 gender-matched controls. The horizontal lines in each figure represent the mean values. *, P = 0.02 as determined by analysis of covariance with age and race as covariates.
Table 1. Effect of therapy on the plasma levels of pro-inflammatory mediators (n=9)

<table>
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<tr>
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<th>Baseline</th>
<th>After therapy</th>
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<tbody>
<tr>
<td>IL-8</td>
<td>6.2 ± 2.0</td>
<td>4.3 ± 1.4 *</td>
</tr>
<tr>
<td>MCP-1</td>
<td>378 ± 62</td>
<td>390 ± 93</td>
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<tr>
<td>C5a</td>
<td>26 ± 15</td>
<td>23 ± 13</td>
</tr>
</tbody>
</table>

Values are means ± standard deviations in pg/ml for the IL-8 and MCP-1 levels and in ng/ml for C5a.
* P < 0.01 as analyzed by Paired t-test.

only measurable in 2 patients (means 13 and 7 pg/ml respectively) and 2 controls (means 114 and 72 pg/ml respectively). As generally known, these cytokines are easily induced by common stimuli and mediate the systemic acute-phase responses, such as fever (31). However, these 4 individuals with detectable IL-1β and TNF-α levels did not display any overt symptoms of infections nor had an enhanced body temperature (data not shown) at the time of the study. The prevalence of IL-6 sero-positivity for patients and controls proved not to be significantly different: IL-6 sero-positivity was observed in 10 out of 19 untreated patients, and in 6 out of 19 controls. From 6 out of 9 the patients, who were IL-6 sero-positive before therapy, only 4 became sero-negative after therapy.

DISCUSSION

The present study showed that the plasma IL-8 levels in untreated periodontitis patients were not different from the levels in plasma of controls without destructive periodontal disease. This finding corroborates the results form a recent study demonstrating that the IL-8 levels in plasma of periodontal maintenance patients did not statistically differ from control subjects (32). However, the present study
additionally showed that reduction or elimination of the periodontal infection resulted in decreased plasma IL-8 levels in the patients. Interestingly, the values after therapy tended to be lower values than the values of the control subjects. This may possibly be explained by the presence of (some) gingivitis in the control group.

The major source of IL-8 production in vivo are the monocytes (33, 34), and the IL-8 levels in plasma correlate with the numbers of peripheral blood monocytes expressing IL-8 mRNA (35). In a previous study from our laboratory, lipopolysaccharide (LPS)-stimulated whole blood cell cultures from the same subjects were analyzed for the release of monocyte-derived IL-8 levels (36). Therefore, it was possible to perform a correlation analysis between the plasma IL-8 levels and the monocyte-derived IL-8 concentrations in the cell cultures. The analysis showed that the IL-8 levels in plasma of all study subjects from the present study correlated significantly with the IL-8 levels in the cell cultures ($r = 0.4, P = 0.002$). This suggests that the IL-8 levels found in plasma are most likely derived from the peripheral blood monocytes.

In line with the analysis for IL-8, the current plasma MCP-1 levels of the study subjects were correlated with the MCP-1 levels in the LPS-stimulated cell cultures from the same subjects (chapter 4). Interestingly, no correlation was found between the MCP-1 levels in plasma and the monocyte-derived MCP-1 levels in the cell cultures. This suggests that other sources in addition to the peripheral blood monocytes contribute to the MCP-1 levels in plasma. It has been shown that vascular endothelial cells produce MCP-1 in response to inflammatory signals and shear stress (37, 38). In addition, endothelial as well as smooth muscle cells in human atheromatous plaques express the mRNA transcript of the protein (39). Furthermore, there are only few cells expressing MCP-1 mRNA in normal arteries, and endothelial cells and subendothelial macrophages seem to be the major source of MCP-1 in early atherosclerotic lesions (39, 40). Therefore, a reported increase in plasma MCP-1 levels with aging, has been suggested to reflect the degree of atherosclerosis (41). To date, it is still not clear whether the chemokine originates form the injured smooth muscle cells or from infiltrating monocytes/macrophages (42). In addition, also the
origin of MCP-1, as an inflammatory marker, in plasma of patients with cardiovascular diseases is unknown, as well as the relationship of plasma and tissue levels of MCP-1 (43).

The present finding that more patients than controls displayed elevated levels of C5a in their plasma indicates that in certain periodontitis patients the complement system is strongly activated, whereas in others not. An explanation for this finding may be that the complement system is activated by systemically released gram-negative bacteria or substances of their membrane intermittently (44). Recently, it has been shown that the incidence of mastication-induced endotoxemia in periodontitis ranges between 16% and 40% and is dependent on the severity and extent of the periodontal infection (12).

In conclusion, after periodontal therapy the plasma IL-8 levels decrease, most likely due to a reduced activation of the peripheral blood monocytes. Systemic MCP-1 levels do not change 3 months after therapy and continue to be higher in periodontitis patients. This could suggest that periodontitis might have contributed to a vascular response, e.g. an inflammation, which is not resolved by periodontal therapy.

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