Susceptibility to periodontitis. Studies with LPS-stimulated whole blood cell cultures
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CHAPTER 4

In Periodontal Disease High Levels of Monocyte-Derived RANTES seem to be Intrinsic, while MCP-1 Levels are Related to the Inflammatory Process

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ABSTRACT

Bacteria colonizing tooth surfaces are essential in the induction of an inflammatory response in the periodontal tissues, but do not cause periodontitis in everyone, implicating differences in the host immune response. These possible differences were studied using lipopolysaccharide (LPS)-stimulated whole blood cell cultures (WBCC), which revealed a down regulation of monocyte derived interleukin-12 (IL-12p70) in untreated periodontitis patients and an up regulation after therapy. IL-12p70 is a crucial factor in the differentiation of Th1 cell responses. Since CC chemokines are able to influence the T cell differentiation via cytokine secretion in antigen-presenting cells, the production of CC chemokines in periodontitis was evaluated. Therefore WBCC were stimulated with LPS from Escherichia coli for 18 hours. Untreated periodontitis patients released 2 fold more RANTES (regulated on activation normal T cell expressed and secreted) and lower levels of IL-12p70 in comparison to controls. A trend towards higher levels of macrophage chemotactrant protein-1 (MCP-1) was also seen in untreated periodontitis patients; while similar levels of monocyte derived chemokine (MDC) and macrophage inflammatory proteins-1α and -1β (MIP-1α and -1β) were found. After periodontal therapy no changes were seen with regard to MDC, MIP-1α, MIP-1β and RANTES, whereas the MCP-1 levels decreased and the IL-12p70 levels strongly increased. The data showed a consistent inverse correlation between the levels of MCP-1 and IL-12p70, suggesting a role for MCP-1 in the regulation of IL-12p70 in periodontitis. The persistent augmented levels of RANTES after therapy may suggest a possible role of this versatile chemokine in the susceptibility to periodontitis.
INTRODUCTION

Periodontitis results from the inflammatory response of the host to the bacterial challenge in the gingival crevicular area (1). Although bacteria are essential in the induction of the inflammatory response in the periodontal tissues, they are insufficient to cause destructive periodontal disease (2). It is generally acknowledged that not everyone is equally susceptible to the disease (1, 2), which implicates intrinsic differences in the host immune response.

In the inflammatory infiltrate, which develops in the periodontal tissues, T- and B-lymphocytes outnumber the APCs, i.e. monocytes, macrophages and dendritic cells (3). Nevertheless, APCs have an important regulatory role in the development of immune responses in general (4). It is commonly acknowledged that inflammatory mediators present at the initiation of the immune response determine the differentiation of Th1 and Th2 cells (5). In this respect IL-12 and PGE$_2$ are important factors: IL-12 induces the development of Th1 cells producing high levels of IFN-γ, while PGE$_2$ either selectively inhibits IFN-γ production directly or indirectly via an IL-12-antagonistic way and thereby favoring the development of Th2 cells (6-8). The knowledge on the role of APCs is based on experiments with the peripheral blood precursors, i.e. the monocytes, either isolated or in WBCC and resembles the in vivo situation (8-10).

Previous experiments in our laboratory with LPS-stimulated WBCC have shown that periodontitis patients release lower levels of IL-12p70 (the biologic active heterodimer of IL-12) and higher levels of PGE$_2$ compared to control subjects, whilst the levels of IL-10 were comparable (11). Interestingly, after periodontal therapy the release of IL-12p70 in LPS-stimulated WBCC from patients strongly increased, whereas the PGE$_2$ to IL-12p70 ratio decreased (Chapter 3). Based on the fact that WBCC stimulated with relatively low concentrations of LPS specifically reflect the behavior of the monocytes (8), it was proposed that APCs from untreated periodontitis patients exhibit a type 2 promoting phenotype, which diminished after
periodontal therapy. Indeed, in untreated periodontal lesions T cells express higher levels of IL-4 and IL-5 mRNA and produce more IL-4 and have a lower IL-2/IL-4 ratio compared to controls (12-14). These observations for periodontal disease are in parallel with other studies that indicate a similar association between Th2 cytokine-biased immunity and a type 2 profile in APCs of patients suffering from HIV infection and atopic allergy (15, 16). Both latter studies show that the monocytic release of IL-12p70 is also lower in patients compared to controls. However, the reasons for this depressed production of IL-12p70 by peripheral blood monocytes in patients with untreated disease is still largely unknown.

Chemokines, well known for their role in directing cell movement, have also been shown to influence the direction of T cell differentiation. A growing body of evidence indicates that CC chemokines have indirect effects on T cell differentiation through their effects on APC trafficking or APC cytokine secretion, as well as direct effects through actions on the differentiating T cell (17). Recently, it has been shown that a selective set of CC chemokines is able to suppress the production of IL-12p70 by human monocytes, and in that way participate in the development of T cell-mediated immune responses (18). Therefore, the present study investigated whether the release of CC chemokines in LPS-stimulated WBCC correlated with the levels of IL-12p70 from periodontally healthy controls and untreated and treated periodontitis patients.

MATERIALS AND METHODS

Study subjects
Nineteen periodontitis patients (mean age 37 years; range 26-45 years), 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. Furthermore, only non-smoking patients were selected, i.e. 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) were recruited from patients of the ACTA referred for the treatment of dental caries. The patient group included 6 subjects with a mixed racial background and the control group 5 subjects. 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.

Eight out of the 19 periodontitis patients volunteered to participate in a specifically designed treatment protocol, including the use of systemic antibiotics. This patient population had a mean age of 34 years (range 26-42 years). Approval by the Institutional Review Board on human studies was obtained and all subjects signed an informed consent.

Clinical procedures
The specifically designed treatment protocol consisted of oral hygiene instructions, full mouth scaling and rootplaning within 24 hours supported by systemic antibiotics for 7 days (Amoxicillin and Metronidazol) and 3 months rinsing with chlorhexidine twice daily. To evaluate the effect of the periodontal therapy, full mouth clinical measurements and microbiological samples at 4 selected sites, i.e. the deepest site in each quadrant of the mouth, were performed before as well as 3 months after therapy. The sampling and culturing procedures were performed in a standardized way, as described previously (19). The presence of A. actinomycetemcomitans, P. gingivalis, B. forsythus, P. intermedia, F. nucleatum, P. micros and C. rectus were determined.

Whole blood cell cultures
Venous blood was collected by venepuncture in sodium heparin-containing blood collecting tubes (VT 100SH tubes; Venoject; Terumo Europe, Leuven, Belgium) of all
subjects participating in both sets of study groups. The blood was diluted five-fold in endotoxin-free RPMI 1640 medium containing L-Glutamine and 25 mM HEPES (Gibco BRL, Life Technologies B.V., Breda, The Netherlands) and was supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. The diluted whole blood was cultured in duplicate in pyrogen-free 24-well flat-bottom tissue culture plates (Costar, Cambridge, MA) in the presence of 1 ng/ml LPS from *E. coli* O127:B8 (Difco, Detroit, MI) for 18 hours at 37°C. The period between the blood collection and the start of the incubation was 1.5 hour. Following culturing, supernatants were stored at -80°C until determination of chemokine and IL-12p70 concentrations. The choice for the non-specific stimulant to activate the WBCC was motivated on the basis of previous experiments, which revealed that not all patients and controls were always colonized by the same major periodontal pathogens. Moreover, since many studies within the field of periodontology and in other areas of research concerning distinct inflammatory diseases, use the common available and highly purified source of *E. coli*-LPS to activate peripheral blood cells (8, 11, 16, 20-23), it facilitates comparisons with other studies. Previous experiments in our laboratory revealed that WBCC stimulation with LPS concentrations of 1 ng/ml was the most optimal for the production of inflammatory mediators, except IL-12p70. The production of IL-12p70 was only measured at 100 ng/ml LPS, since this cytokine is released in extreme low levels in < 100 ng/ml LPS stimulated WBCC. Furthermore, the produced dose response curves did not differ between patients and controls as well as for patients before and after periodontal therapy.

Venous blood of all participants was also collected in an EDTA(K3) containing tube (Becton Dickinson Vacutainer System Europe, Meylan, France) for the determination of the leukocyte count and differentiation, and were performed in standard automated procedures.
Assays
All used specific monoclonal antibodies, neutralizing antibodies and the recombinant human macrophage chemoattractant protein (MCP)-1, monocyte derived chemokine (MDC), macrophage inflammatory protein (MIP)-1α, MIP-1β and regulated on activation normal T cell expressed and secreted (RANTES) were purchased from R&D Systems (Abington, UK). Measurements of the WBCC supernatants were performed by specific solid-phase sandwich ELISA, according to the manufacturers protocol. Interference of plasma and heparin in the ELISAs was prevented by the use of a high performance ELISA buffer developed at the CLB (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands). The bounded chemokines were detected using streptavidin-poly horseradish peroxidase conjugate (CLB, Amsterdam, the Netherlands) and tetramethylbenzidine/hydrogen peroxide as a substrate (Biosource Europe SA, Nivelles, Belgium). The detection levels of the assays were 10 pg/ml for MCP-1, 20 pg/ml for MDC, and 30 pg/ml for MIP-1α, MIP-1β and RANTES. A specific solid-phase sandwich ELISA was also used for the measurement of the IL-12p70 levels, as described previously (8). The detection limit of the IL-12p70 assay was 3 pg/ml. The chemokine and cytokine concentrations of the duplicate cultures all ranged within the normal intra-assay variability.

Statistical Analysis
SPSS package (version 10.0 for Windows, Chicago, IL, USA) was used for data analysis. Data analyses of the log-transformed chemokine and cytokine concentrations were performed by means of an analysis of covariance. The number of monocytes was used as a covariate, as were the gender, age and race of the study subjects. P-values < 0.05 were considered significant.
RESULTS

IL-12p70 and CC chemokine production in periodontitis

Some CC chemokines may inhibit the production of IL-12p70 (18), therefore the present study measured the production of several CC chemokines and IL-12p70 simultaneously in WBCC and evaluated their possible correlations. Since LPS-stimulated WBCC specifically reflect the behavior of the monocytes (8) and activated monocytes do secrete the chemokines under study (24), the chemokine and IL-12p70 concentrations are expressed per number of monocytes (Fig. 1 and 2). There were no differences found in the average number and percentage of the monocytes between the patients and controls, and also not in patients before and after therapy (data not shown).

In Figure 1A the released levels of IL-12p70 in the LPS-stimulated WBCC are presented and show for the untreated patients a lower IL-12p70 production in the cultures compared to the controls. With regard to the levels of the MIP-1β, MDC and MIP-1α in the supernatants there were no differences between untreated patients and control subjects (Fig. 1B and C). However, the untreated patients proved to produce almost two fold more RANTES in comparison with the control group (29 ng versus 15 ng/10^6 monocytes, P = 0.01). It was remarkable that within the untreated periodontitis group there were patients who produced up to six and eight fold higher levels of RANTES in comparison to the controls. Furthermore, the MCP-1 levels in the blood cell cultures from the untreated periodontitis patients showed a trend towards higher values in comparison with the controls (P = 0.07). In 7 out of the 19 untreated patients the released concentrations of MCP-1 in the WBCC were two to four fold higher compared to the cultures of the controls. In addition, the released levels of MCP-1 in the LPS-stimulated cultures correlated with the released levels of IL-12p70 in the same cultures (r = -0.3, P = 0.02). This indicated a reversed relationship between the production of the CC chemokine MCP-1 and the levels of
Fig. 1. LPS-induced production of IL-12p70 (A) and the CC chemokines MIP-1β, MDC, MCP-1, MIP-1α, and RANTES (B and C) in 19 untreated periodontitis patients and 19 controls. The whole blood cell cultures were stimulated with 1 ng/ml LPS from *E. coli* for 18 hours, while the cultures for IL-12p70 were stimulated with 100 ng/ml LPS. Data are means ± SEM and are expressed as IL-12p70 or CC chemokine production per 10⁶ monocytes. The individual data per subject were the mean of two different cultures. *P < 0.05*, as determined by analysis of covariance with gender, age, race and number of monocytes as covariates. □, controls; ■, patients.

IL-12p70 in the WBCC: higher levels of MCP-1 correlated with lower levels of IL-12p70.
**IL-12p70 and CC chemokine production after periodontal therapy**

The extent and severity of the inflammation in the periodontal tissues from the 8 patients, who participated in the treatment protocol, is clinically expressed as high percentages of bleeding on probing and deep probing depths (Table 1). The aim of periodontal therapy is to restore periodontal health, which is characterized clinically by shallow probing depths and low bleeding scores and microbiologically by a low percentage of periodontal pathogens (25). As can be seen from Table 1 the mean percentage of shallow probing depths increased after periodontal therapy and the mean percentage of deep probing depths reciprocally decreased. On average the bleeding on probing scores decreased from 80% to 20% after therapy. The major periodontal pathogens *A. actinomycetemcomitans*, *P. gingivalis* and *B. forsythus* were no longer detectable after periodontal therapy (data not shown).

**Table 1.** The extent and severity of the inflammatory process in the periodontal tissues before and after therapy (n=8)

<table>
<thead>
<tr>
<th>Baseline (mean)</th>
<th>After therapy (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD</td>
<td></td>
</tr>
<tr>
<td>1 – 4 mm</td>
<td>41 %</td>
</tr>
<tr>
<td>≥ 5 mm</td>
<td>59 %</td>
</tr>
<tr>
<td>BOP</td>
<td>all sites</td>
</tr>
</tbody>
</table>

*PD = probing depths, recorded to the nearest millimeter*  
*BOP = bleeding on probing.*

In order to analyze whether the lower production of IL-12p70 in untreated periodontitis patients is an intrinsic characteristic of their monocytes or acquired as a result of the disease state itself, the production of IL-12p70 was evaluated before and after periodontal therapy.
Fig. 2. LPS-induced IL-12 and CC chemokine production in 8 periodontitis patients before and after periodontal therapy. For the measurements of IL-12p70 (A) the whole blood cell cultures were stimulated with 100 ng/ml LPS from *E. coli* for 18 hours, whereas the cultures for the CC chemokines (B and C) were stimulated with 1 ng/ml LPS from *E. coli*. Data are means ± SEM and are expressed as IL-12p70 or chemokine production per 10^6 monocytes. The individual data per subject were the mean of two different cultures. * P < 0.05, as determined by analysis of covariance for matched pairs with the number of monocytes as a covariate. ■, before therapy; □, after therapy.

Similar analyses were performed for the release of the selected CC chemokines. After therapy the levels of IL-12p70 released in the cultures of the periodontitis
patients increased on average 2.5 fold (Fig. 2A).

Interestingly, these levels were now comparable to the levels seen in the control group and statistical testing showed no difference anymore between treated patients and controls ($P = 0.7$, compare Fig. 2A with Fig. 1A). This suggests that the lower levels of IL-12p70 production in untreated periodontitis patients is not an intrinsic characteristic of their monocytes, but is most likely acquired by the chronic presence of the disease. For the selected set of CC chemokines, only the levels of MCP-1 changed after periodontal therapy, whereas the levels of MIP-1β, MDC, MIP-1α as well as RANTES were unaffected (Fig. 2B and C).

Table 2. The individual proportional changes in the release of MCP-1, IL-12p70 and the clinical inflammatory response after therapy

<table>
<thead>
<tr>
<th>Patient</th>
<th>MCP-1</th>
<th>IL-12p70</th>
<th>BOP</th>
<th>PD ≥ 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-47 %</td>
<td>147 %</td>
<td>-81 %</td>
<td>-98 %</td>
</tr>
<tr>
<td>2</td>
<td>-27 %</td>
<td>81 %</td>
<td>-92 %</td>
<td>-85 %</td>
</tr>
<tr>
<td>3</td>
<td>-43 %</td>
<td>75 %</td>
<td>-84 %</td>
<td>-89 %</td>
</tr>
<tr>
<td>4</td>
<td>-67 %</td>
<td>314 %</td>
<td>-92 %</td>
<td>-79 %</td>
</tr>
<tr>
<td>5</td>
<td>-24%</td>
<td>227 %</td>
<td>-79 %</td>
<td>-86 %</td>
</tr>
<tr>
<td>6</td>
<td>-41%</td>
<td>97 %</td>
<td>-86 %</td>
<td>-70 %</td>
</tr>
<tr>
<td>7</td>
<td>9 %</td>
<td>2 %</td>
<td>-37 %</td>
<td>-89 %</td>
</tr>
<tr>
<td>8</td>
<td>32 %</td>
<td>-4 %</td>
<td>-74 %</td>
<td>-47 %</td>
</tr>
</tbody>
</table>

BOP = bleeding on probing
PD = probing depths, recorded to the nearest millimeter.

Table 2 shows that all but 2 treated patients had decreased levels of MCP-1 after therapy and concomitant increased levels of IL-12p70. Moreover, just the 2 patients who showed an increase in the levels of MCP-1 had in fact no change in the IL-12p70 levels after periodontal therapy. This suggests again, a consistent and inverse
relationship between the released levels of MCP-1 and IL-12p70 in the cell cultures. The same 2 patients with increased levels of MCP-1 after therapy also showed less favorable outcomes of therapy, i.e. more residual inflammation (Table 2).

DISCUSSION

The present data revealed that in the case-control experiments the production of RANTES was higher and the levels of IL-12p70 lower in periodontitis patients, while the levels of MDC, MIP-1α, and MIP-1β were comparable between subject groups. The values of MCP-1 were somewhat higher in the patients than in controls and correlated with the decreased production of IL-12p70 in the same cultures. Periodontal therapy substantially reduced the clinical inflammatory process in the periodontal tissues and resulted in decreased levels of MCP-1 and increased levels of IL-12p70 in the stimulated cell cultures. The levels of MDC, MIP-1α, MIP-1β and RANTES were unaffected after therapy. These results suggest that MDC, MIP-1α and MIP-1β have, at least in E. coli LPS-stimulated WBCC, no particular relationship with periodontitis.

The suppressive effect of MCP-1 on IL-12 was first shown in mice. The treatment of mice with MCP-1 resulted in a reduced production of IL-12p70 within mucosal tissues (26). In addition, in a mouse model of endotoxic shock, the exogenous administration of MCP-1 significantly protected mice from endotoxin-induced lethality (27). The protection correlated with low IL-12p70 levels in serum and resembled the protection observed after anti-IL-12p70 treatment (27). Recently, MCP-1 has been shown to suppress the production of IL-12p70 in activated human monocytes, while other chemokines, such as MIP-1α and RANTES, seem to have no or little effect (18). The results of the present study are in line with this latter finding that monocyte-derived MCP-1 molecules most likely suppress the levels of IL-12p70 in peripheral blood monocytes. The release of MCP-1 in the WBCC from untreated periodontitis
patients tended towards higher levels, while the production of IL-12p70 was reduced. Moreover, the levels of MCP-1 correlated inversely with the levels of IL-12p70 in the cultures from untreated patients and controls. In addition, the present study also demonstrated that the levels of MCP-1 reduced significantly after periodontal therapy whilst the levels of IL-12p70 increased. Therefore the lower IL-12p70 levels in untreated periodontitis patients are transient in nature and correlate strongly with the changed levels of MCP-1 in the WBCC. Consequently, the levels of MCP-1 and IL-12p70 reflected the clinical improvements in periodontal inflammation.

Although the levels of MCP-1 and IL-12p70 correlated inversely, the percentage increase in IL-12p70 after therapy was 2 to 10 fold more than the percentage decrease in MCP-1. This may implicate that other factors could also be responsible for the suppression of IL-12p70. For example, IL-10 and PGE₂ are well known inhibitors of the production of monocyte derived IL-12p70 (20, 28). It has been suggested that the suppression of IL-12p70 by MCP-1 is partly due to the production of IL-10 by the stimulated monocytes, but not PGE₂ (18). However, this was not confirmed when multiple donors were used, as there was a significant interdonor variation in this effect (18). Above that, previous experiments with LPS-stimulated WBCC showed that there were neither differences between periodontitis patients and controls in the levels of IL-10 nor between patients before and after periodontal therapy (11, chapter 3).

The consistently higher production of RANTES in periodontitis patients both before and after therapy, suggests an intrinsic characteristic of monocytes from these patients, and might be an important susceptibility factor for periodontitis. To date, very little is known about the possible role of RANTES in the pathogenesis of periodontitis and limited and inconclusive data are available (29-31). Although RANTES is a potent chemokine for the selective attraction of monocytes, memory T cells, eosinophils and basophils (32-34), it is also able to activate leukocytes of the lymphoid origin (35). RANTES may engage the T cell receptor complex, since a direct correlation between a RANTES-induced response and CD3 expression has been shown (36). Furthermore, RANTES has been shown to up-regulate the
expression of co-stimulatory molecules on T cells and APCs (37, 38). Therefore, it might be suggested that the high concentrations of RANTES released by APCs from periodontitis patients could additionally stimulate T cells in the periodontal lesion without the presence of antigen. This non-specific T cell activation could be an explanation for the general belief that bacteria alone are not enough to cause destructive periodontal disease (39). Since IL-12p70 is obligatory in directing the development of Th1 cell responses (5), and the APC derived levels of IL-12p70 were lower in untreated periodontitis patients, it may be postulated that APCs from untreated periodontitis patients have a depressed type 1- or a more type 2-inducing phenotype compared to controls. Therefore, it can be further postulated that RANTES may also contribute to the development of such Th2 responses in the periodontal lesion without the necessity of antigen presentation. Indeed, it is generally believed that Th2 cells dominate in the periodontal lesion, whereas plasma cells are the most active secretory cells (40, 41).

In conclusion, it may be suggested that due to the chronic inflammation in the periodontal tissues peripheral blood monocytes have up-regulated levels of MCP-1 and consequently, most likely in an autocrine or paracrine way, down-regulated IL-12p70 levels, which are restored again after periodontal therapy. In addition, it may be suggested that intrinsic high levels of RANTES can be regarded as a possible susceptibility factor for periodontitis.

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