Susceptibility to periodontitis. Studies with LPS-stimulated whole blood cell cultures
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CHAPTER 5

The Long-Term Effect of Full Mouth Tooth Extraction on the Responsiveness of Peripheral Blood Monocytes; a Case Report

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ABSTRACT

Since after periodontal therapy some inflammation will always remain, the present study investigated the long-term effect of full mouth tooth extraction therapy on the host immune response in a case with generalized terminal adult periodontitis, to further explore the intrinsic or acquired nature of this response. Before and 3, 9, 20 and 32 months after therapy venous blood was collected. Total and differential white blood cell counts were determined and whole blood cell cultures were incubated with lipopolysaccharide to stimulate the production of inflammatory mediators in monocytes. After full mouth tooth extraction, the numbers of total peripheral white blood cells and neutrophils decreased over time. The release of the chemokines IL-8 and macrophage chemoattractant protein-1 (MCP-1) decreased two-fold over time, whereas no changes were found for the other studied cytokines, chemokines and prostaglandin E$_2$. On the basis of previous studies and the present case, the higher expression of IL-8 and MCP-1 in peripheral blood monocytes from periodontitis patients is most likely acquired, as their levels decreased over time when the periodontal infection is controlled. Since both chemokines are regarded as important factors related to atherosclerosis, the possible connection between periodontitis, IL-8, MCP-1 and atherosclerosis are discussed.

INTRODUCTION

It is commonly accepted that, mainly Gram-negative, bacteria accumulating on the tooth surfaces, play an essential role in the pathogenesis of periodontitis. In fact, periodontitis is the result from the interaction of the host immune cells and these Gram-negative bacteria, or their cell wall components like LPS (1, 2). However, despite the colonization with periodontal pathogens not everyone is equally susceptible to periodontitis (2). This implicates that possible differences in the
immune responses of the host may exist. To study potential differences in the immune capacity, WBCC stimulated with LPS have frequently been used (3-7). It has been clearly shown that LPS-stimulated WBCC reflect the behavior of the monocytes when relatively low levels of LPS are employed (3, 8, 9, 10). Furthermore, peripheral blood monocytes represent the role of APCs, i.e. monocytes/macrophages and dendritic cells (11, 12). APCs in turn are acknowledged as important regulators of the specific immune response (13). In this respect, inflammatory mediators such as cytokines, prostaglandins and chemokines released by APCs are crucial in the development of specific T-helper cell responses (14-18).

The reported differences in the responses of peripheral blood monocytes between periodontitis patients and control subjects (7, 19, 20) may reflect either intrinsic or acquired differences in cell activity. Therefore recent experiments evaluated the effect of non-surgical periodontal therapy on the responsiveness of peripheral blood monocytes in LPS-stimulated WBCC (chapters 3 and 4). These experiments revealed selective changes in the production of monocyte-derived inflammatory mediators after extensive periodontal therapy including the use of systemic antibiotics. Although the periodontal condition improved substantially after the non-surgical periodontal therapy, no complete resolution of the disease process was possible. Still about 10% of the sites displayed deepened probing depths and 20 to nearly 30% of the sites showed bleeding on probing (chapters 3 and 4). This residual periodontal infection could still have impact on the responsiveness of peripheral monocytes. In order to exclude any influence of the periodontal infection on the responsiveness of the peripheral blood monocytes, and therefore to better discern the acquired or intrinsic nature of the different responses in periodontitis patients, the inflamed periodontium has to be completely eliminated. This can be achieved by a full mouth tooth extraction therapy. The present case report studied the responsiveness of peripheral blood monocytes before and during a period of 3 years after full mouth tooth extraction therapy in a patient with generalized terminal adult periodontitis.


MATERIALS AND METHODS

Patient

A 43-year-old Caucasian male with generalized terminal adult periodontitis was referred to the Department of Periodontology of the Academic Center for Dentistry Amsterdam (ACTA) for diagnosis and treatment. The patient's natural dentition consisted of 26 teeth with the 14, 17, 35, 37, 45 and 47 missing. Intra-oral examination revealed heavy amounts of dental plaque and calculus, generalized gingival recession, probing pocket depths > 10 mm at all teeth, generalized mobility and bleeding on probing with suppuration and in all molars a class III furcation involvement. Furthermore, dental radiographs showed that all teeth exhibited bone loss extending beyond ¼ of the root length, while the majority of the teeth displayed bone loss extending to the apices of the roots. Due to the hopeless condition of all teeth, periodontal therapy was impossible. Since the only remaining treatment option was extraction of all teeth, the patient was asked to participate in the present study.

The patient was a former heavy smoker (15 pack years) and had ceased smoking 12 years ago. The patient was in good general health and free from any systemic disease and use of medication. Furthermore, the patient reported no clinical symptoms of bacterial, viral or parasitic infections at any time point of the study. Approval by the Institutional Review Board on human studies was obtained and the patient signed an informed consent.

Whole blood cell cultures

Peripheral blood samples of the patient were obtained before the full mouth tooth extraction and 3, 9, 20 and 32 months after therapy. At each time point, except at 9 months due to technical problems, two separate blood collections were performed with one-month interval and were considered replicates. The blood samples were collected by venepuncture in sodium heparin-containing blood collecting tubes (VT 100SH tubes; Venoject; Terumo Europe, Leuven, Belgium). 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 non-pyrogenic 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) (7) for 18 hours at 37°C. Previous experiments had shown that the used LPS concentration of 1 ng/ml was the most optimal for the stimulation of the WBCC. Since the production of the biologically active heterodimer of the cytokine IL-12 is low in LPS-stimulated WBCC, IL-12p70 was measured in cultures stimulated with concentrations of 100 ng/ml LPS. The period between the blood collection and the start of the incubation was 1.5 hour. Supernatants were stored at -80°C until determination of cytokine, PGE$_2$ and chemokine concentrations.

Peripheral blood was also collected in an EDTA(K$_3$) containing tube (Becton Dickinson Vacutainer System Europe, Meylan, France) for the determination of the total and differential white blood cell counts, which were performed in standard automated procedures.

**Assays**

The levels of the cytokines IL-1β, IL-6, IL-8, IL-10 and TNF-α were measured in the supernatants 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. The sensitivity of the kits for the various cytokines used varied from 4 to 9 pg/ml. Measurements of IL-12p40 and IL-12p70 levels were performed by a specific solid-phase sandwich ELISA as described previously (9). The IL-12p70 ELISA detects biologically active IL-12. The detection limit of the IL-12 assays was 20 pg/ml for IL-12p40 and 3 pg/ml for IL-12p70. PGE$_2$ concentrations were determined using the ACE™ competitive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI), which had a detection limit of 15 pg/ml.
Measurements of the levels of CC chemokines in the WBCC supernatants were also performed by specific solid-phase sandwich ELISAs. All used specific monoclonal antibodies, neutralizing antibodies and the recombinant human MCR1, MD), MIP-1α, MIP-1β and RANTES were purchased from R&D Systems (Abington, UK), and the ELISAs were performed 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 (Amsterdam, NL). The bound chemokines were detected using streptavidin-poly horseradish peroxidase conjugate (CLB, Amsterdam, NL) and tetramethylbenzidine as a substrate (Biosource Europe SA, Nivelles, B). The detection limits of the assays were 10 pg/ml for MCR1, 20 pg/ml for MDC, and 30 pg/ml for MIP-1α, MIP-1β and RANTES.

**Statistical analysis**

SPSS package (version 10.0 for Windows, Chicago, IL, USA) was used for data analysis. The cytokine, PGE$_2$ and chemokine concentrations were log-transformed. The two measurements, one month apart, were considered as independent replicates. The change over time, from $t = 0$ up to 32 months, was evaluated by means of a one-way analysis of covariance with time as a factor and the number of monocytes as a covariate. Similar analyses were performed for the peripheral blood cell numbers. $P$-values < 0.05 were considered significant.

**RESULTS**

**White blood cell counts before and after extraction**

Peripheral blood was collected for the determination of the total and differential white blood cell counts before and 3, 9, 20 and 32 months after full mouth tooth extraction. The total number of peripheral white blood cells decreased within 3 months after tooth extraction and then stabilized or slightly increased, as did the number of neutrophils. The mean values for the total white blood cell numbers were 4.52 x 10$^6$/L.
after extraction and $3.76 \times 10^9/L$ after extraction therapy; those for the number of neutrophils were $2.93 \times 10^9/L$ before extraction and $2.37 \times 10^9/L$ after. No changes over time could be assessed for the other white blood cell numbers (Table 1).

Table 1. The total and differential white blood cell counts ($10^9/L$) before and 3, 9, 20 and 32 months after full mouth extraction

<table>
<thead>
<tr>
<th></th>
<th>WBC</th>
<th>neutrophils</th>
<th>eosinophils</th>
<th>basophils</th>
<th>lymphocyte</th>
<th>monocytes</th>
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<tbody>
<tr>
<td>Before</td>
<td>4.42</td>
<td>2.86</td>
<td>0.12</td>
<td>0.02</td>
<td>0.99</td>
<td>0.32</td>
</tr>
<tr>
<td>4.62</td>
<td></td>
<td>2.99</td>
<td>0.15</td>
<td>0.02</td>
<td>1.06</td>
<td>0.29</td>
</tr>
<tr>
<td>After</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.30</td>
<td>2.12</td>
<td>0.09</td>
<td>0.03</td>
<td>0.84</td>
<td>0.22</td>
</tr>
<tr>
<td>3.60</td>
<td></td>
<td>2.19</td>
<td>0.10</td>
<td>0.01</td>
<td>1.04</td>
<td>0.24</td>
</tr>
<tr>
<td>9</td>
<td>3.60</td>
<td>2.26</td>
<td>0.15</td>
<td>0.03</td>
<td>0.89</td>
<td>0.28</td>
</tr>
<tr>
<td>20</td>
<td>4.22</td>
<td>2.53</td>
<td>0.09</td>
<td>0.03</td>
<td>1.14</td>
<td>0.27</td>
</tr>
<tr>
<td>4.00</td>
<td></td>
<td>2.55</td>
<td>0.14</td>
<td>0.02</td>
<td>0.96</td>
<td>0.33</td>
</tr>
<tr>
<td>32</td>
<td>3.80</td>
<td>2.31</td>
<td>0.12</td>
<td>nd</td>
<td>0.97</td>
<td>0.40</td>
</tr>
<tr>
<td>3.80</td>
<td></td>
<td>2.60</td>
<td>0.09</td>
<td>nd</td>
<td>0.89</td>
<td>0.23</td>
</tr>
</tbody>
</table>

P-value: $0.02$ 0.03 0.4 0.8 0.6 0.7

WBC, the total number of the peripheral white blood cells.
nd, not determined.
At all listed time points two separate blood collections with one-month interval were performed, while at 9 months after therapy blood samples were only once collected due to technical problems.
* as determined by one-way analysis of variance on all individual data.

**Cytokine, PGE₂ and chemokine production before and after extraction**

Peripheral blood was also collected for the WBCC experiments. The cultures were incubated with LPS to stimulate the production of inflammatory mediators in monocytes. At all time points, except 9 months, two separate WBCC with one-month interval were incubated and the production of inflammatory mediators measured. The analysis showed no change over time for the release of IL-1β, TNF-α, IL-10, PGE₂,
IL-6, IL-12p40 and IL-12p70 in the LPS-stimulated WBCC after the full mouth tooth extraction therapy (Figures 1 and 2). However, for the chemokine IL-8 a consistent decrease was found over time (Figure 2B), resulting nearly 3 years after baseline into a two-fold reduction. The levels of the CC chemokines MIP-1α, MIP-1β, RANTES and MDC in the WBCC did not change over time (Figure 3). The levels of the CC chemokine MCP-1 however decreased consistently over time (Figure 3A). Nearly 3
years after the complete elimination of periodontal infection the MCP-1 levels were two-fold reduced in comparison with baseline.
Fig. 3. The production of CC chemokines in LPS-stimulated whole blood cell cultures. LPS from *E. coli* was used in a concentration of 1 ng/ml and stimulated the cultures for 18 hours at 37°C. The supernatants were measured for the concentrations of MCP-1 (A), MIP-1α (B), MIP-1β (C), RANTES (D) and MDC (E). The individual data on each time point were the mean of two separate cultures. *P*-values as determined by one-way analysis of covariance with time as a factor and the number of monocytes as a covariate.
DISCUSSION

The present case report tried to explore over a 3-year period whether the responsiveness of immune cells may be an acquired phenomenon by the periodontal infection or is an intrinsic characteristic of these cells. In a case with generalized severe terminal adult periodontitis the effect of full mouth tooth extraction on the white blood cell counts and on the responsiveness of peripheral blood monocytes were longitudinally evaluated. After the complete elimination of the periodontal infection, i.e. after full mouth tooth extraction, the number of peripheral blood neutrophils was decreased. It is well known that the numbers of peripheral blood neutrophils increase with infection, since they are rapidly mobilized to inflamed tissues and live shortly (23). Indeed, it has been shown that periodontitis as well as experimental gingivitis is associated with increased numbers of peripheral blood neutrophils (24-26). Therefore, elimination of the periodontal infection should result in lower numbers of circulating neutrophils, as was found after periodontal therapy (27) as well as in the present case.

After the complete elimination of the periodontal infection, the monocyte-derived releases of IL-8 and MCP-1 consistently decreased over a time period of nearly 3 years. With regard to the other studied inflammatory mediators, no statistical changes could be demonstrated in this single case. In previous reports on periodontal treatment (3 months follow-up), it was also found that the levels of MCP-1 decreased, while a statistically significant decrease of the IL-8 levels could not be detected (chapters 2 and 3). The reason for the unchanged IL-8 levels 3 months after periodontal therapy could be that the measurements were performed at a relatively early time point after therapy, since it appeared in the present case that reduction developed gradually over time. Furthermore, in a previous case control study it was shown that the levels of PGE$_2$ were higher in untreated periodontitis patients compared to control subjects, whereas the levels of IL-12p70 were lower (7). In the periodontal treatment study, however, it was found that 3 months after periodontal therapy the PGE$_2$ levels showed a trend towards reduction and the levels
of IL-12p70 strongly increased (chapter 3). Most likely, due to the limitations of the statistical analysis (the experimental material consisted of 1 single case), these reported short-term changes in the levels of PGE₂ and IL-12p70 could not be detected in the present case report. On the whole, the current conclusions are based on findings in a single patient and it is uncertain as yet whether similar patients react in the same way.

IL-8 belongs to the CXC subgroup of the chemokine family, structurally indicating the presence of an amino acid (X) that separates the first two of four-conserved cysteine residues (C), and specifically chemo-attracts neutrophils. MCP-1 belongs to the CC subgroup, which structurally lacks an amino acid separating the first two cysteine residues, and specifically chemo-attracts monocytes (28). Both, IL-8 and MCP-1, are important mediators of inflammatory processes and the host defense, and play an important role in a variety of diseases (29, 30). Since the periodontal infection pursues when not treated, circulating leukocytes will continuously be chemo-attracted to the site of inflammation. Among these, the monocytes have an important secretory function through the production of inflammatory mediators that directs the immune response. Most likely, the peripheral blood monocytes are primed by soluble factors derived from the periodontal infection. Consequently, after full mouth tooth extraction therapy this priming may no longer occur, which might be reflected by a lower production of IL-8 and MCP-1 in LPS-stimulated WBCC. This latter phenomenon might have systemic consequences, as will be discussed below.

MCP-1 and IL-8 are acknowledged to play a major role in the pathogenesis of subclinical atherosclerosis (31). MCP-1 as well as IL-8 have been shown to cause rapid adherence of monocytes onto vascular endothelium, whereas related chemokines did not (32). Moreover, migration of monocytes into the blood vessel wall is one of the earliest events in the pathogenesis of atherosclerosis and MCP-1 seems to be an important chemokine involved in the monocyte infiltration during atherosclerosis (31). Furthermore, in human atheromatous plaques, enhanced levels of MCP-1 and IL-8 are found (33, 34). The direct evidence for the pivotal role of MCP-1 in the pathogenesis of atherosclerosis has come from knockout mice studies.
Either mice lacking the receptor for MCP-1 (CCR2) or being deficient for the MCP-1 protein have decreased atherosclerosis, while mice over-expressing MCP-1 by monocytes/macrophages show accelerated atherosclerosis (35-37). The association of cardiovascular diseases and periodontitis has been reported, as well as the presence of inflammatory, cardiovascular disease associated, factors, e.g. C-reactive protein and fibrinogen, in the serum of humans and non-human primates with periodontitis (26, 38, 39). However, this periodontitis-cardiovascular disease relationship has concentrated on the clinical cardiovascular events and not on subclinical atherosclerosis. Recently, a study showed that periodontitis is associated with more atherosclerosis in the wall of the carotid artery in humans(40). It has been postulated that the relationship between chronic infections in general and cardiovascular diseases in particular are systemically induced immune responses that elevate the levels of established risk factors for atherosclerosis (41). In case of the periodontal infection, it may be that periodontitis primes circulating monocytes towards increased IL-8 and MCP-1 expression, which subsequently promotes the infiltration of monocytes into the blood vessel wall. Within the vessel wall the inflammatory response is potentiated and consequently, the atherosclerotic lesion formation aggravated (35).

In conclusion, the elimination of the periodontal infection results in a decreased number of circulating neutrophils and in a depressed responsiveness of peripheral blood monocytes, in terms of IL-8 and MCP-1. This lower expression of IL-8 and MCP-1 after extraction therapy suggests that the altered responsiveness of monocytes from periodontitis patients is more likely an acquired phenomenon rather than an intrinsic characteristic. Additionally, it is suggested that the increased expression of MCP-1 and IL-8 in untreated periodontitis may contribute to the acceleration of the atherosclerosis process.
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