The Effect of Periodontal Treatment on IL-6 Production of Peripheral Blood Monocytes in Aggressive Periodontitis and Chronic Periodontitis Patients

Mehrdad Radvar[1,2]*, Jalil Tavakkol-Afshari[3], Mahboobeh N. Bajestan[1], Mohammad-Reza Naseh[4], Hamid-Reza Arab[1]

[1]Department of Periodontology, School of Dentistry, [2]Mashhad Dental Research Centre, [3]Immunology Research Center, Mashhad University of Medical Sciences, Mashhad, [4]Department of Periodontology, School of Dentistry, Ghazvin University of Medical Sciences, Ghazvin, Iran

Abstract

Background: Several cytokines, including IL-6 have been implicated in the pathogenesis of periodontal disease. It is established that monocytes from periodontitis subjects show an increased production of IL-6 as compared to healthy subjects. However, little is known about the effect of periodontal treatment on IL-6 production by monocytes in subsets of periodontitis patients. **Objective:** The aim of the present study was to evaluate the effect of surgical periodontal treatment on IL-6 production of peripheral blood monocytes (PBM) in aggressive periodontitis patients (AP) and chronic periodontitis patients (CP) before and after stimulation by *E.coli* LPS. **Methods:** Fifteen AP patients, 15 CP patients and 15 periodontally healthy subjects (PH) took part in the study. PBM IL-6 production was measured, using ELISA, before and after stimulation of cultured PBM cells by 0.1 μg/ml LPS of *E.coli*. Following full-mouth non-surgical and surgical periodontal treatment of the AP and CP groups, the same measurements were repeated for these two groups. **Results:** LPS-stimulated IL-6 production was significantly greater than non-stimulated IL-6 for all 3 groups. Before periodontal treatment, LPS-stimulated IL-6 production of the AP group was significantly greater than the other 2 groups. Periodontal treatment did not result in a significant decrease in unstimulated or LPS-stimulated IL-6 production by PBM cells in AP and CP patients. No correlation was detected between IL-6 levels and baseline clinical parameters or changes in clinical parameters. **Conclusion**: PBM cells in AP patients might be hyper-responsive in terms of IL-6 production. This hyper-responsiveness does not seem to return to that of healthy subjects even after a successful periodontal treatment. Moreover, the regulation of host inflammatory mechanisms upon LPS challenge might be different between AP and CP patients.

Keywords: Periodontitis, Monocytes, IL-6

*Corresponding author: Dr. Mehrdad Radvar, Mashhad Dental School, Mellat-Park Square, Mashhad University of Medical sciences, Mashhad, Iran. P.O. Box: 91735, Tel: (+) 98 915 3134629, Fax: (+) 98 511 8829500, e-mail: radvarm@mums.ac.ir

INTRODUCTION

Periodontitis is an inflammatory disease of the supporting structures of teeth and ultimately leads to bone destruction, tooth mobility and tooth loss if left untreated. Periodontitis is a multifactorial disease with dental plaque as the primary etiological factor and many putative risk factors (1). Aggressive periodontitis (AP) is a severe form involving 7–14% of the population (2). Dental plaque is required for the initiation of the disease, but it seems that it is the quality of host response which determines, to a large extent, the severity and extension of tissue destruction (1).

It is generally believed that LPS from Gram-negative bacteria of dental plaque penetrate the periodontal tissues and activate immune and inflammatory cells (1).

Host cells such as blood monocytes and tissue macrophages become activated by the LPS and release pro-inflammatory cytokines and prostaglandins. To study potential differences in immune capacity, blood cell cultures stimulated with LPS have been used (3-8). It has been reported that responses of peripheral blood monocytes between periodontitis and normal subjects may be different (7, 9,10). This difference may be either intrinsic or acquired. Cross-sectional studies might distinguish differences in monocytic responses between periodontitis and healthy subjects (11-13), but these studies could not provide insight as to whether the hyper-responsiveness of monocytes in periodontitis patients is intrinsic or extrinsic.

In order to recognize the acquired or intrinsic nature of the difference between periodontitis patients and periodontaly healthy subjects, the effect of periodontal treatment on responsiveness of monocytes must be studied. Gustafsson et al reported that cultured monocytes from successfully treated chronic periodontitis subjects released larger levels of IL-1β than healthy controls (8). However, no comparison between pre- and post-treatment levels was made. Fokkema et al monitored the effect of full-mouth extraction in a single patient. IL-8 and MCP-1 showed a gradual decrease over time. Other cytokines including IL-6 did not change (14). Fokkema et al reported no change in IL-1β, IL-10, TNFα, PGE2, IL-6, IL-8, and IL-12p40 of monocytes after periodontal non-surgical therapy. Nevertheless, a significant increase occurred in IL-12p70 (15). In another study of effect of non-surgical treatment on cultured monocyte responsiveness, Fokkema et al reported no change in the levels of “regulated upon activation, normal T cell expressed and secreted (RANTES)" (16). The authors concluded that RANTES was intrinsically elevated in periodontitis patients. In the aforementioned studies no distinction was made between CP and AP and all patients were categorized as "periodontitis subjects". Moreover, except in one single case report (14), the periodontal infection was not resolved. This was shown by residual bleeding on probing (BOP) of 28% and pocket prevalence (4mm or greater) of 20% (15). No data has been reported to date on the effect of full-mouth periodontal surgery on responsiveness of monocytes.

Inflammatory and immune host cells release inflammatory mediators and cytokines in response to bacterial challenge. This may result in the destruction of periodontal tissues (17). Interleukin-6 (IL-6) is a pro-inflammatory cytokine which has been found in gingival crevicular fluid and gingival tissues of periodontally diseased dentition. Its level has been reported to have close correlation with attachment loss and bone resorption over time (18). Macrophages are the main source of IL-6 in inflammatory infiltrate (19).

In a previous study on an Iranian population, we found that consistent with studies on Caucasians, blood monocytes from AP patients release larger amounts of IL-6 upon stimulation by LPS than healthy controls (13).We report here on longitudinal differences between CP, AP and healthy subjects. The aim of this study was to determine the

effect of periodontal surgical pocket elimination on responsiveness of cultured peripheral blood monocytes as measured by unstimulated and LPS-stimulated release of IL-6 in aggressive periodontitis and chronic periodontitis patients.

MATERIAL AND METHODS

Study Design and Patient Selection. In our longitudinal study, 15 subjects with AP (67% female) and 15 patients with CP (60% female) and 15 periodontally healthy subjects (60% female) were selected from patients referred to Mashhad Dental School, Iran, for dental treatment. The two patient groups underwent a full course of cause-related non-surgical treatment plus quadrant surgical pocket elimination where needed. Patients with remaining pocketing (≥5mm) and BOP following the non-surgical treatment were scheduled for surgical treatment only if their plaque levels were less than 15% (20). Prior to the start of treatment and two months following the last surgical visit, periodontal clinical measurements were recorded and a 15 ml blood sample was taken from each subject for cell culture. The control group provided blood samples only at baseline. The criteria for AP and CP were based on the "International Workshop for Classification of Periodontal Disease" (21). The patients of AP group had clinical and radiographical evidence of periodontal attachment loss in multiple sites, and were < 35 years old. Furthermore, the degree of periodontal destruction was not commensurate with their plaque levels. CP patients included patients of > 40 years with clinical and radiographic evidence of periodontal disease. The degree of periodontal destruction among these patients was consistent with the presence of abundant plaque and calculus. The control group was periodontally healthy and showed no sign of periodontal destruction or gingivitis. Subjects with systemic diseases, including known infectious or inflammatory disorders, and subjects who had received antibiotics or periodontal treatment during the past six months were excluded. The subjects were all non-smokers. The Ethics Committee of Mashhad University of Medical Sciences approved the study and informed consent was obtained from all subjects.

Clinical Measurements. At baseline and two months following the end of the last surgical treatment, the patients in AP and CP groups were subjected to full-mouth periodontal examinations by a single examiner whose intra-examiner reliability had been proved acceptable in a pilot study. These included probing pocket depth, clinical attachment level and the presence of bleeding on probing (BOP) at 6 sites around each tooth using a calibrated periodontal probe with William's markings (Hufridy, USA). Third molars were excluded.

Cell Culture and IL-6 Measurement. A 15ml blood sample was obtained by venipuncture from each subject in tubes containing 10% EDTA. Mononuclear cells were immediately isolated with the use of Ficoll-Hypaque density gradients, and centrifuged three times, at 2000 rpm for 20 min, 1500 rpm for 5 min, and 1500 rpm for 5 min. The viability of the cells used throughout the experiment was always 95% as determined by the Trypan blue exclusion test. Monocytes were isolated from the rest of the mononuclear cells by their ability to adhere to the glass plate wall of the culture dish after incubation in RPMI-1640 medium (Gibco, Ireland) at 37°C in 5% CO_2 for 24 hours. The media were supplemented with 200 U/ml penicillin, 2 g/l streptomycin, and 10% FBS. This was then verified by light microscopic observation of the cell shape. The monocytes were cultured in six-well culture plates (NUNC, Denmark) at a concentration of 1.6×10^5 cells/ml. LPS from *E. coli* (Sigma, MO, USA) was added at 0.1 μg/ml. This concentration was selected from 3 concentrations of 0.01, 0.1 and 1 μg/ml

since a pilot study on 2 subjects in our laboratory revealed that this produced the maximum IL-6 release from monocytes. After six hours, 500 µl of supernatant from both unstimulated and LPS-stimulated cell culture wells were collected and transferred to micro tubes and kept at –20°C until analysis using the ELISA (Enzyme- Linked Immunosorbent Assay) technique. The LPS from *E.coli* does not differ significantly from the LPS of the periodontal pathogen *P.gingivalis* in cytokine release in cell culture (12). Supernatants were diluted 10 times for ELISA. A commercially available ELISA kit for human IL-6 (BenderMed System, Vienna, Austria) was used for analysis. Preliminary studies showed that almost all IL-6 release took place within the first 6 hours of incubation.

Statistical analysis. The Wilcoxon test was used to compare the pre- and post-stimulation IL-6 production levels in each group. The Kruskal-Wallis test was used to compare IL-6 levels between the 3 groups in unstimulated and LPS-stimulated states. If a significant difference was detected, then Mann-Whitney U test was used to detect the significantly different groups. The change in the clinical parameters across the two periodontitis groups as a result of treatment were analyzed using paired t-tests. A P.value <0.05 was considered as significant. The data were analyzed using SPSS version 11.5 software.

RESULTS

Table 1 shows the clinical parameters and their changes throughout the study period in the AP and CP subjects. PPD and CAL decreased significantly after surgery among both groups of periodontitis subjects. Furthermore, the percentage of sites with BOP as well as the percentage of sites with remaining PPD of greater than 4mm decreased significantly among both groups. Table 2 shows IL-6 levels in peripheral blood monocyte culture among the three categories of subjects before and after surgery in both unstimulated and LPS-stimulated states. At baseline, the LPS-stimulated IL-6 levels among AP patients was significantly greater than healthy controls (p=0.04) and CP patients (p=0.03). The unstimulated IL-6 levels in cell culture of CP patients after treatment decreased compared to baseline and the levels in AP patients remained higher than baseline, although neither difference was significant. However the levels of unstimulated IL-6 post treatment of AP patients were significantly greater than those of CP patients. After therapy, the LPS-stimulated monocyte cultures of AP and CP patients showed an elevated response compared to healthy controls. However, the response in AP patients was not greater than the baseline stimulated response. No significant correlation was detected between IL-6 levels and baseline clinical parameters or changes in clinical parameters.

Table 1. Clinical parameters and their changes throughout the study period among aggressive periodontitis (AP) and chronic periodontitis (AC) subjects (mean ± standard deviation)

Clinical parameter		Baseline	After surgery	Change	P.value
Probing depth (mm)	AP	4.52±1.04	1.63±0.18	2.89±1.02	<0.001
	CP	3.07±0.51	1.79±0.42	1.28±0.67	<0.001
Attachment level (mm)	AP	5.75±1.37	3.84±1.02	1.91±1.01	<0.001
	CP	3.89±1.19	3.60±1.02	0.29±0.49	<0.001
% Bleeding on probing	AP	92.93±10.22	23.18±9.29	69.75± 15.45	<0.001
	CP	73.64±16.22	19.92±8.20	53.72± 17.65	<0.001
% pockets > 4mm	AP	54.74± 16.88	3.35±1.93	51.39± 16.69	<0.001
	CP	27.12± 12.46	3.42±3.66	23.70± 12.99	<0.001

Table 2. IL-6 concentration in peripheral blood cell culture in different categories of patients and stimulation states at baseline and after treatment

Category		Baseline		After treatment	
		Unstimulated (µg/ml)	LPS-stimulated	Unstimulated	LPS-stimulated
Healthy	Mean	417.98± 115.97	908.10±275.31	NA	NA
	Median	285.64	412.55		
Aggressive periodontitis	Mean	473.12±153.78	1703.41±247.62	796.89ý±616.45	1515.97 ý±257.01
	Median	171.60	1698.62	682.00	1243.60
Chronic Perio-dontitis	Mean	649.11±203.31	993.13±219.19	305.86 ý ±423.75	1428.57ý±274.58
	Median	321.00	856.00	31.00	1648.00
P. value		0.521	0.041*	0.008§	0.716
		Kruskal-Wallis test	Kruskal-Wallis test	Mann Whitney-U test	Mann Whitney-U test

*Significant difference between aggressive periodontitis group versus healthy group (p=0.036, Mann Whitney U test), and between aggressive periodontitis group and chronic periodontitis group (p=0.026, Mann Whitney U test).
ý Not significantly different from baseline corresponding figures.
§ Significant difference between aggressive periodontitis group and chronic periodontitis group.

DISCUSSION

No previous data has been reported on the effect of periodontal surgery on the responsiveness of human peripheral blood monocytes. In the present study the effect of full-mouth periodontal surgery on IL-6 release by peripheral blood monocytes was evaluated in order to explore the acquired or intrinsic nature of functional differences of these cells in periodontitis patients. Our data indicates that monocytes from AP and CP patients show different behaviors as a result of periodontal treatment. In a previous study, we showed that AP patients produce larger amounts of IL-6 than healthy controls when stimulated by *E.coli* LPS (13). The present study confirms this finding and shows that baseline levels of IL-6 from AP were higher than those of CP and healthy controls under stimulation with *E. coli* LPS. This might be due to the fact that the AP patients had a worse disease than CP patients, but also might be due in part to hyper-responsive monocytes.

After treatment, all clinical parameters significantly improved and the percentage of pockets > 4 mm were less than 4% of all sites among both groups of patients. In other words there was little residual disease. Nevertheless, two months following the completion of treatment, IL-6 production from the stimulated monocytes of both AP and CP patients were increased compared to healthy subjects. This is in line with the study of Gustafsson et al who found a significantly greater IL-1β production from monocytes of treated periodontitis subjects as compared to healthy controls (8). It is consistent with the concept of a hyper-responsive monocytic trait among both CP and AP periodontitis patients on contact with bacterial plaque stimulants such as LPS. On the other hand, after treatment unstimulated monocytes of AP patients produced significantly more IL-6 than both unstimulated CP and unstimulated healthy controls. Also, the LPS-stimulated IL-6 levels in AP patients did not increase as compared to baseline. Although the increase in the LPS-stimulated IL-6 level in CP patients following therapy did not reach statistical significance, the increase was still remarkable. This may indicate that in the AP group the monocytes are intrinsically hyper-responsive, but in the CP patients the monocytes have to be primed. This occurs as a result of the transient inoculation effect of mechanical treatment of periodontitis which produces a heightened monocyte response in CP patients when they are subsequently challenged with bacterial plaque toxins. In our study, the time interval between the last surgery and the second blood sampling was 2 months. It may be that the IL-6 levels need a longer period to return to normal. Fokkema et al demonstrated that RANTES was intrinsically elevated in

LPS-stimulated monocytes of periodontitis patients and did not change following treatment (16). Likewise, in a separate study the LPS-stimulated RANTES levels did not change following non-surgical therapy (14). In the two mentioned study, however, no distinction was made between AP and CP patients. Our data indicated that in AP patients, in the absence of any stimulation, the IL-6 levels did not drop as a result of elimination of bacterial plaque, implicating an intrinsically elevated response. These observations are, in fact, consistent with the clinical observation of lower tolerance to plaque among AP patients. Even after successful treatment of periodontal disease, meticulous plaque control and rigid maintenance program is crucial for long-term success. In other words, it could be stated that both normal and successfully treated periodontitis patients will keep their healthy periodontium in the absence of plaque, but the successfully treated patients will probably have greater risk of recurrence of disease if plaque control is not ideal. The threshold for harmful plaque is lower in susceptible patients. Since the monocytes from such treated patients, even in the absence of LPS stimulation, release greater amounts of pro-inflammatory cytokines such as IL-6, it would be conceivable that these levels could more easily surpass the "threshold level" to trigger tissue destruction if stimulated by bacterial plaque.

Only a single case report has been published on the long-term effect of full-mouth tooth extraction on monocytes (15). The level of IL-6 did not show any change, but IL-8 and MCP-1 showed a gradual decrease up to 3 years post-treatment. Such a study design with long-term follow-up and virtually absolute eradication of infection would be an ideal study design to evaluate the behavior of blood monocytes after periodontal treatment provided that it is performed on sufficient number of subjects.

This was a study with a relatively small sample size. Similar studies with larger sample size are warranted to further explore differences among various categories of periodontitis in terms of monocyte inflammatory response to bacterial endotoxin.

We used *E.coli* LPS to stimulate monocytes. It could be argued that the use of LPS from periodontal pathogens such as *P.gingivalis* would probably be more relevant, although it has been shown that LPS from *E.coli* and *P.gingivalis* do not differ significantly in cytokine release in cell culture (13). Furthermore, the experiments on IL-6 release from cultured monocytes were performed once for each sample. If duplicate IL-6 measurements rather than single measurements had been taken, probably a narrower error margin could have appeared on the data with more clear-cut differences.

This was an *in vitro* study. *In vitro* studies, although easier to control, could not precisely mimic the *in vivo* situation. Complex interactions within the inflammatory cellular network and the interplay of several cytokines perhaps could produce a scenario somewhat different from *in vitro* experimental settings. Nevertheless these results clearly indicate that the behavior of peripheral blood monocytes might be different among periodontitis groups. In conclusion, the data of this study indicate that AP patients may have a monocytic hyper-responsiveness trait that predispose to inflammatory reactions in response to LPS challenge even after a successful periodontal treatment. Moreover, it seems that the regulation of host inflammatory mechanisms upon LPS challenge might be different between AP and CP patients.

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