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The association between neutrophil numbers and interleukin-1α concentrations in gingival crevicular fluid of smokers and non-smokers with periodontal disease

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Abstract

Objectives: To test whether neutrophil numbers are directly correlated with interleukin-1α (IL-1α) concentrations in gingival crevicular fluid (GCF) of patients with periodontitis, and to investigate the effects of smoking on these parameters.

Materials and Methods: A total of 99 GCF samples from 33 patients (14 smokers) suffering from severe chronic periodontitis were collected using Durapore™ filter strips. Polymorphonuclear leucocyte (PMN) numbers were counted using a Coulter cell counter and IL-1α levels were determined by ELISA. Total GCF protein was measured by Bio-Rad assay as a surrogate measure of GCF volume.

Results: Mean IL-1α concentrations were significantly reduced in smokers compared with non-smokers (non-smokers: 3.29 ± 2.02 pg/μg protein, smokers 1.59 ± 1.13 pg/μg protein). There was no association between PMN numbers and IL-1α concentrations found when analysed either by site or by patient. PMN numbers were not significantly different between the two groups (non-smokers: $1.16 \times 10^6 \pm 1.04 \times 10^6$; smokers: $7.30 \times 10^5 \pm 8.07 \times 10^5$). Smoking did not affect mean total protein concentration of samples.

Conclusions: Smoking significantly decreased IL-1α concentrations in GCF without affecting GCF volume sampled. The lack of association between IL-1α concentration and neutrophil numbers suggests that the reduced IL-1α concentrations seen in smokers is independent of any possible effect of smoking on neutrophil chemotaxis, and further suggests that smoking may directly inhibit IL-1α production.

Key words: cytokines; gingival crevicular fluid; neutrophils; periodontitis; smoking

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Interleukin-1 (IL-1) is one of the major proinflammatory cytokines involved in the pathogenesis of periodontitis (Page 1991, Tatakis 1993). It has wide ranging effects including the regulation of inflammatory mediators such as other cytokines and PGE2, it can regulate adhesion molecule expression in endothelial cells, and has a strong catabolic role in periodontal disease by inducing the formation and activation of osteoclasts from bone marrow precursors and by stimulating the release and activation of matrix metalloproteinases (MMPs) by macrophages and other cells.

IL-1 is present in two active isoforms: α and β. Together with the competitive antagonist IL-1ra these proteins are encoded for by adjacent genes (IL-1A, IL-1B, IL-1RN) in the IL-1 gene cluster. Recent studies have reported associations between a variety of polymorphisms in the IL-1 gene cluster and increased risk of periodontal disease (Kornman et al. 1997, McDevitt et al. 2000, Parkhill et al. 2000, Cullinan et al. 2001). Some of the disease-associated

alleles have been shown to be associated with increased IL-1 protein production, suggesting that a possible mechanism for this increased risk is the genetic control of IL-1 production resulting in increased IL-1-induced tissue damage (Pociot et al. 1992, Shirodaria et al. 2000). IL-1α and β are found at elevated concentrations in gingival crevicular fluid (GCF) of patients with periodontitis, although these concentrations may vary widely between different subjects.

There are a number of possible sources of IL-1 in the GCF. These include production by macrophages and other cells within the periodontal tissues and subsequent passsage into the pocket, or, as neutrophils may also be a source of IL-1, the local production and release of IL-1 from neutrophils within the pocket itself (Matsuki et al. 1993, Liu et al. 2001). Neutrophils are the predominant type of polymorphonuclear leucocyte (PMN) and are the first line of defence of the periodontal tissues (Van Dyke & Vaikuntam 1994). They rapidly accumulate in the infected periodontal tissues in large numbers and migrate into the pocket to combat the microorganisms. They kill bacteria by phagocytosis and extracellular killing using both oxidative and non-oxidative mechanisms. However, this function can also frequently result in damage to the adjacent tissues and excacerbation of the periodontal tissue damage, so-called ''by-stander'' damage.

Tobacco smoking is a well-recognised risk factor for periodontitis, and in addition is known to markedly impair healing responses post-treatment (Haber & Kent 1992, Grossi et al. 1994, Haber 1994, Kaldahl et al. 1996, Tonetti 1998). It is thought that the mechanisms that connect smoking and periodontitis include the relative inhibition of normal host immune and inflammatory responses, which may be particularly evident on neutrophil function. (Pabst et al. 1995, Numabe et al. 1998, Ryder et al. 1998a, b, 2002).

In a previous study investigating the effect of the transpositional polymorphism at position –889 of the IL-1A gene on IL-1α concentrations in GCF, we found that IL-1α concentrations were consistently low in heavy smokers irrespective of IL-1 geneotype (Shirodaria et al. 2000). Given that IL-1α is a cytokine, which is largely cell membrane-associated rather than secreted (Dinarello 1989), these observations might simply be a direct reflection of neutrophil concentration in GCF, combined with reduced neutrophil numbers in the pockets of smokers with periodontal disease, rather than a direct effect of smoking on IL-1α production per se. Consequently, the aims of the study reported here were to test the association between PMN numbers and IL-1α concentrations in GCF of patients with periodontal disease, and to investigate the effect of smoking on PMN numbers in GCF of these patients.

Materials and Methods

Patient selection

The study received ethical approval from the East London and City Health Authority local research ethics committee. Subjects were recruited from patients newly referred to the Periodontal Consultant Clinic at the Royal London Hospital. They were systemically healthy, aged from 42–55 years and suffering from severe chronic periodontitis (at least eight teeth with pocket >6 mm measured using an Ash 14W periodontal probe). Individuals who had a history of antibiotic or periodontal treatment within 6 months from the sampling time, or suffered from systemic diseases (e.g. diabetes mellitus, immunosuppresive illness, blood dyscrasias) or medications (e.g. cyclosporin, phenytoin or Ca channel blockers) that would affect the clinical presentation of the disease were excluded from the study. Sites with evidence of suppuration or clinical or radiographic evidence of endodontic pathology were also excluded from the study. All subjects were given written instructions on the procedures prior to obtaining informed consent.

Sampling

GCF from three to five pockets measured at greater than 6 mm from each patient was sampled by means of Durapore™ (Millipore Ltd, Watford, UK) hydrophilic membrane filters of polyvinilidene di-fluoride with pore size of 0.22 μm as previously described (Andersen & Cimasoni 1993). Strips were cut to 2×8 mm size. The gingivae were dried by air and cotton pellets 1 min before sampling and the area isolated by means of cotton rolls. The strip was placed in the pocket until mild resistance to further apical movement was felt and then left in place for 10 s. The sample strip was then inserted into plastic sealable eppendorf tubes contaning 200 μl of a balanced isotonic salt solution (Isoton, Coulter Electronics Ltd, Luton, UK). Samples contaminated by any signs of blood were rejected. Samples were processed within 2 h of sampling time. All samples were stored separately and used for site-specific measurement of IL-1 and PMN numbers.

Sample analysis

Prior to analysis the sample tubes were vigorously shaken for 15 s using a vortex mixer. Fifty μl of the sample solution was used to determine numbers of neutrophils using a Coulter Cell Counter. The thresholds for particles size were 6–16.2 μm. Three consecutive measurements were taken for each sample and a mean was calculated.

The rest of the sample was then stored at -70°C until further analysis. At analysis samples were defrosted and 50 μl was taken for measurement of protein concentration by Bio-Rad colorimetric protein assay. According to the principle of this technique the binding of the acidic dye solution Coomassie Brilliant Blue G-250 with the proteins in the GCF samples results in a qualitative colorimetric reaction that can be read in a microplate reader at 595 nm, and compared against a standard curve of bovine albumin of known concentrations.

A further 50 μl was used for assessment of the IL-1α concentration by means of enzyme-linked immunoabsorbant assay (Biotrak ELISA, Amersham, UK) according to the manufacturer's protocol.

Reliability of the cell counting

In order to check whether the Durapore™ strips were able to retrieve PMNs reliably from the GCF additional samples were taken and processed for histological examination. Four strips were used, of which two were vortexed for 15 s while the remaining two were not vortexed. They were fixed with 10% formol saline, embedded in paraffin wax and separate wax blocks were prepared, each containing one strip. Seven-micrometer sections were prepared, stained with haematoxylin and Giemsa dye and examined under the microscope.

Data analysis

Site-specific raw data for PMN counts, protein concentration and IL-1α were

converted to absolute values present in the original GCF sample by correction for dilutions used during sampling and analysis. PMN and IL-1α concentrations were calculated by dividing these figures by the total protein present and expressing results as cells/μg total protein and pg/μg total protein, respectively.

Mean data were also calculated from samples from each patient. Statistical analysis was calculated using GraphPad™ Prism software. Data for both total IL-1 and PMNs, and concentrations were analysed. Mean values and standard deviations were calculated and statistical differences between smoker and non-smoker groups were tested by the Mann–Whitney *U*-test using the mean data for each patient, so as to use the patient as the unit of analysis. The association between PMN numbers and IL-1α concentration was tested for all sites by determining correlation coefficients.

Results

Patient data

A total of 33 subjects were recruited (28 females and 16 males). Their mean age was 46.9 years (range 42–57 years). Fourteen were smokers and 19 were non-smokers or ex-smokers, having stopped smoking at least 2 years prior to sampling time. The mean pocket depth of the sampled sites was 7.5 mm and ranged from 6 to 10 mm. In all, 99 sites were sampled, 45 from smokers and 54 from non-smokers.

Reliability of cell sampling

Histological examination of the sections prepared from the Durapore™ strips before and after vortexing showed many haematoxylin and Giemsa-stained cells adherent to the freshly collected strips. After vortexing the strips were seen to be almost entirely free of cell deposits, demonstrating the efficacy of the vortex mixing to shake the cells into suspension in the buffer solution (not shown).

Correlation between PMN numbers and IL-1α concentrations

Fig. 1 shows the plot of PMN numbers against IL-1α concentrations for all individual sites. There was no association found between PMN numbers and IL-1α concentration, either when analysed as one group ($R^2 = 0.0047$, $p = 0.51$) or when data were separately analysed for smokers and non-smokers (smokers $R^2 = 0.007$, $p = 0.59$; non-smokers $R^2 = 0.0038$, $p = 0.66$).

Patient data for smokers and non-smokers

The mean results from each individual patient are summarised in Table 1. The total protein present in each GCF sample was determined as a surrogate measure of the volume of the GCF in each sample. There was no significant difference between total protein in samples obtained from smokers ($10.45 \pm 6.7\ \mu g$) and those from non-smokers ($11.56 \pm 4.8\ \mu g$; $p = 0.17$ the Mann–Whitney test). There was a wide range of results for both PMNs and IL-1α amongst patients (Table 1), and indeed between individual sites from the same patient (data not shown). The mean concentration of IL-1α in smokers was less than half of that seen in non-smokers (smokers 1.59 ± 0.3 pg/μg protein; non-smokers 3.29 ± 0.46 pg/μg protein; $p = 0.0074$, the Mann–Whitney test).

There was no significant difference in total PMN numbers between smokers and

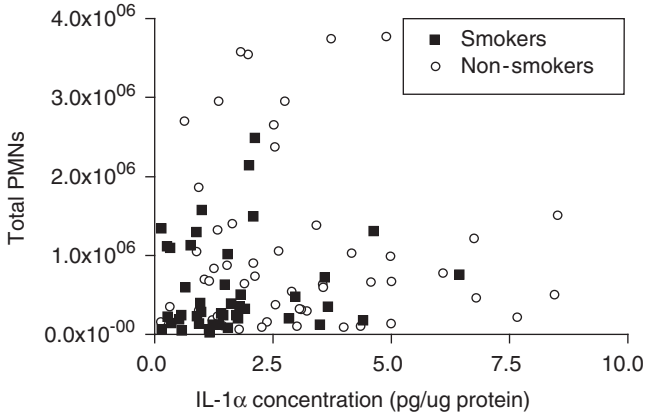

Fig. 1. Correlation between PMN numbers and IL-1α concerntration in individual sites from non-smokers and smokers. There was no association seen in either non-smokers ($R^2 = 0.048$; $p = 0.12$) or smokers ($R^2 = 0.005$; $p = 0.78$).

Table 1. Summary data of mean values for each patient of PMN numbers, IL-1α (pg) and total protein (μg)

Smokers			Non-smokers		
PMNs	Total protein	Total IL-1α	PMNs	Total protein	Total IL-1α
3,252,000	7.04	14.01	2,435,733	16.16	32.48
1,303,467	7.13	7.47	814,400	10.15	47.65
642,133	6.22	16.36	935,467	10.75	78.49
418,133	4.02	18.57	1,989,600	9.33	41.48
282,800	5.63	14.14	1,868,400	16.88	38.35
347,200	7.11	13.12	598,933	17.30	47.54
1,120,533	6.07	4.12	3,371,200	10.25	47.31
683,200	13.69	7.84	482,133	10.92	48.26
852,000	13.80	7.24	1,038,400	9.24	38.34
240,800	8.19	7.82	649,600	5.37	10.15
100,000	8.46	5.02	1,752,000	9.34	19.00
354,800	20.63	19.65	307,200	17.13	15.71
416,320	10.19	9.70	881,600	24.05	23.50
206,400	28.16	19.71	237,867	8.07	3.28
			2,080,533	13.28	17.22
			492,400	9.81	42.06
			542,933	3.59	9.24
			454,400	10.88	19.90
			164,000	7.16	19.82
Mean: 729,985	10.45	11.77	1,110,358	11.56	31.57
SD: 806,702	6.70	5.43	883,456	4.88	18.64

non-smokers (smokers $7.30 \pm 8.07 \times 10^5$; non-smokers $11.1 \pm 8.83 \times 10^5$; $p=0.11$). Mean PMN *concentrations* were nearly identical in both groups (smokers $10.1 \pm 11.45 \times 10^4$ cells/μg protein, non-smokers $9.8 \pm 5.5 \times 10^4$ cells/μg protein).

There was no significant association between total IL-1α and total protein in patient mean samples in either smokers or non-smokers (smokers $R^2 = 0.14$; $p = 0.19$; non-smokers $R^2 = 0.02$; $p = 0.5$; Fig. 2). Similarly, no correlation was found between PMN numbers and total protein concentration in either smokers ($R^2 = 0.057$; $p = 0.41$) or non-smokers ($R^2 = 0.026$; $p = 0.51$) (Fig. 3).

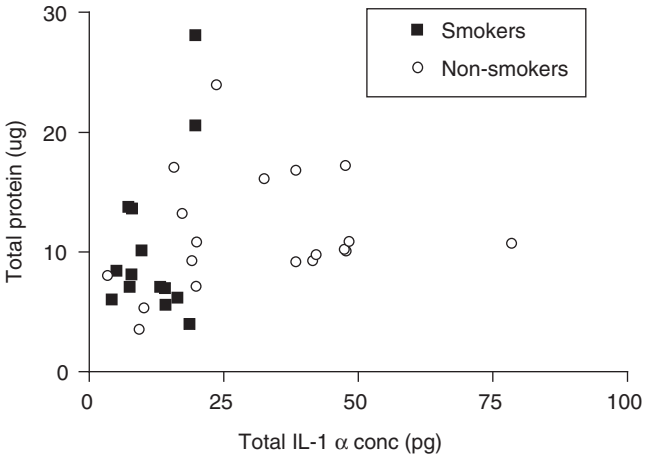

Fig. 2. Correlation between IL-1α concentration and total protein in GCF. Mean values for individual patients. There was no association seen ($R^2 = 0.006$; $p = 0.5$).

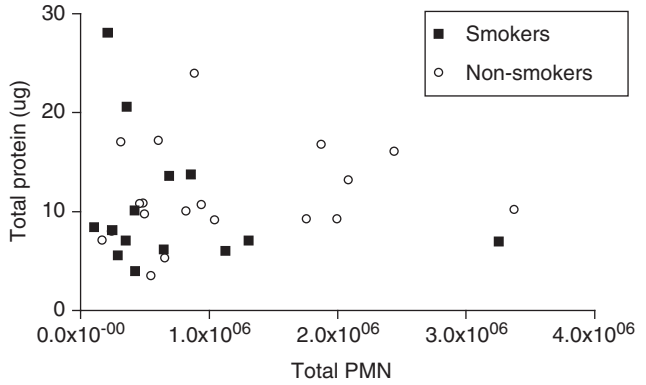

Fig. 3. Correlation between PMN numbers and total protein in GCF in non-smokers and smokers. Mean values for individual patients. There was no association seen in either non-smokers ($R^2 > 0.001$; $p = 0.96$) or smokers ($R^2 = 0.1$; $p = 0.18$).

Discussion

The role of IL-1 in the pathogenesis of periodontitis has been well described (Page 1991, Tatakis 1993). Although most of the studies to date have particularly focussed on the possible role of IL-1β, IL-α has similar biological activities to IL-1β, binds to the same receptors with similar affinity, and studies demonstrate that IL-α is present, at least in GCF, at similar concentrations to IL-1β (Masada et al. 1990). There has been increasing interest in the possible role of IL-1 as a determinant of periodontal disease susceptiblity with the publication of a number of recent studies demonstrating a link between IL-1 genotype and risk of periodontal disease (Kornman et al. 1997, McDevitt et al. 2000, Parkhill et al. 2000, Cullinan et al. 2001). These studies suggest the possibility that these putative genetic risk factors may exert their effects on disease by regulating the amount of IL-1 production during inflammation (Pociot et al. 1992, Shirodaria et al. 2000). The sources responsible for the production of IL-1 in GCF have not been fully determined. It is known that many cell types including macrophages, keratinocytes and neutrophils can produce IL-1 during inflammation. It is possible that the IL-1 concentrations present in GCF may be due to diffusion of IL-1 produced by macrophages within the tissues into the periodontal pocket (Matsuki et al. 1993), or alternatively may largely reflect that which is produced locally within the pocket by the neutrophils. This might be expected to be particularly the case for IL-1α, which is principally cell-associated rather than secreted in soluble form.

Cigarette smoking is known to be a major risk factor for severe periodontitis (Grossi et al. 1994, Haber 1994, Tonetti 1998). However, the precise mechanisms of action of smoking on periodontal disease are not fully understood. The importance of neutrophil function as the first line of defence against periodontal disease has often been commented on, for example, Van Dyke & Vaikuntam (1994). Many studies have assessed the effects of smoking on the neutrophil function. However, the effect of smoking by-products on the numbers of neutrophils in the pocket environment has not been thoroughly investigated. Pauletto reported lower neutrophil numbers in smokers (Pauletto et al. 2000) while Persson suggested that smoking had no effects on neutrophil numbers in periodontitis patients (Persson et al. 2001). However, both studies referred to oral rather than pocket neutrophils. Recent studies in vitro provide further data that smoking may inhibit neutrophil chemotaxis, but this remains to be demonstrated in the clinical situation (Ryder et al. 2002).

In a previous study we reported a reduced IL-1α concentration in GCF in smokers irrespective of IL-1 genotype suggesting an anti-inflammatory effect in smokers (Shirodaria et al. 2000). Possible explanations for this observation included a general reduction in inflammation in smokers, reduced neutrophil numbers in GCF resulting in decreased total local production, or a direct suppression of IL-1α production as a result of smoking. Thus in the present study we investigated this further by testing the effects of smoking on GCF volume, IL-1α concentration and PMN numbers in subjects with severe periodontal disease. We believe this is the first reported study where PMN numbers have been directly compared with IL-1α concentrations in GCF.

The requirement for recovering both the cellular and fluid phases of a GCF sample presents a number of problems. The sampling method needs to be able to recover both phases of the GCF and we also needed to then be able to recover cells to be able to count them electronically. In order to do this we used the Durapore™ strip method originally described and tested by Andersen & Cimasoni (1993). These hydrophilic strips are able to collect GCF readily but, most importantly, allow subsequent elution of both the cells and fluid by a simple vortexing method. In the present study we processed additional strips for histology to confirm the validity of the method in support of the previous work. It was not possible to determine directly the volume of the GCF samples collected, by Periotron determination, which requires the placement of the filter strip into the beaks of the machine for assays, as it is highly likely that this would have disrupted the cells adherent to the filter and invalidated the results. Thus, in an attempt to overcome this we determined total protein content of each sample as a surrogate measure of GCF volume. In support of this approach total GCF protein is known to vary with inflammation and sampling time in a similar pattern to GCF volume (Hattingh & Ho 1980, Curtis et al. 1988, 1990). In determining concentrations total IL-1α was divided by total protein to express concentrations as pg/μg total protein.

The main positive finding of this study was the marked reduction in IL-1α concentration seen in smokers compared with non-smokers. This observation of lower IL-1α concentration supports our previous observations (Shirodaria et al. 2000). There was no significant difference in PMN numbers seen in smokers, although the total number of patients in this study was small, raising the possibility of a type II error. Indeed if PMN numbers are analysed on a site-specific basis the reduction in PMN numbers in smokers is statistically significant (data not shown). Smoking did not affect total protein in GCF, and by inference did not affect GCF volume. Taken together these data suggest that smoking may have specific actions on the inflammatory process but does not have a general effect in suppressing inflammation. In support of this, the lack of correlation between total IL-1α and total protein in individual samples suggests that IL-1α production is not simply regulated by degree of inflammation and is likely to be affected by many different complex mechanisms, which might include IL-1 genotype.

The reduced IL-1α concentration seen in smokers is consistent with the hypothesis that IL-1α concentration reflects the local production of IL-1α by neutrophils in the periodontal pocket. However, this hypothesis is not supported by the fact that no correlation was seen between PMN numbers and IL-1α concentration from individual sites. This was the case even when data for smokers and non-smokers were analysed separately. The data also suggest the likelihood that the concentration of IL-1α in GCF is not principally the result of production locally by neutrophils, and that the GCF levels are a reflection of IL-1α production in the inflamed subjacent gingival tissues.

The other striking observation from the data in the study is the very wide range of data for both PMN numbers and IL-1α concentration from different sites, often even from different sites in the same subject. These observations are consistent with data from previous studies of both GCF neutrophil numbers and IL-1α concentrations (Masada et al. 1990, Zappa et al. 1992). This variation is likely to reflect a whole range of factors including genetically determined individual variations, the site-specific nature of the disease and the variation in inflammatory status of sites with time. This observation emphasises the value of the approach used in the present study for analysis of both cellular and fluid phases of the same GCF sample.

Periodontitis is generally regarded as a hyperinflammatory condition, and the production of IL-1 is associated with activation of a range of mechanisms of tissue damage including macrophage activation, MMP production and bone resorption. Thus the finding of decreased IL-1α in smokers in this study is perhaps counter-intuitive given their predispostion to increased periodontal destruction. However, it is well recognised that clinical levels of inflammation and gingival bleeding are often reduced in smokers compared with non-smokers (Biddle et al. 2001). Studies in vitro have found that extracts of tobacco smoke can inhibit cytokine production by peripheral blood moncytes including of IL-1β (Ouyang et al. 2000). In contrast to the findings with IL-1α reported here, other recent studies have not found an effect of smoking on IL-1β and IL-1RA levels in GCF (Bostrom et al. 2000, Giannopoulou et al. 2003), although the latter study demonstrated elevated levels of IL-6 and IL-8 in smokers. The data overall suggest that smoking has the capacity to deregulate normal homeostatic mechanisms of cytokine production in a variety of ways.

In summary, the results of the study here demonstrate that IL-1α concentration in GCF is significantly reduced in smokers but there was no correlation between PMN numbers and IL-1α. These results suggest that smoking affects PMNs and IL-1α production by different mechanisms, which are not simply an overall anti-inflammatory effect, and further support the idea that much of the IL-1α present in GCF is derived from the inflamed tissues rather than being produced locally by neutrophils in the pocket. Further studies would be valuable to investigate the molecular mechanisms of cytokine production regulation including the influences of genetic factors and tobacco smoking.

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