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Progression of periodontal disease and interleukin-10 gene polymorphism

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Background and Objective: Interleukin-10 is a key immunoregulatory cytokine that may be of significance in the immunopathogenesis of chronic inflammatory diseases such as periodontal disease. Molecular genetic studies have defined a number of haplotypes that may be associated with differing levels of interleukin-10 secretion. The present study investigated the possible association between interleukin-10 gene polymorphism and periodontal disease progression.

Material and Methods: Genomic DNA was obtained from 252 adults who were part of a prospective longitudinal study on the progression of periodontal disease in a general adult Australian population. Single nucleotide polymorphisms at positions −592 and −1082 in the interleukin-10 promoter were analysed using an induced heteroduplex methodology and used to determine interleukin-10 promoter haplotypes in individual samples. Periodontitis progression was assessed by measuring probing depths and relative attachment levels at regular intervals over a 5-year period. A generalized linear model was used to analyse the data, with age, gender, smoking status, interleukin-1 genotype and *Porphyromonas gingivalis* included as possible confounders.

Results: There was a significant ($p \approx 0.02$) main effect of interleukin-10 haplotypes, with individuals having either the ATA/ACC or the ACC/ACC genotype experiencing around 20% fewer probing depths of ≥4 mm compared to individuals with other genotypes. Age and smoking had significant ($p < 0.001$) additional effects.

Conclusion: These data suggest that the interleukin-10 genotype contributes to the progression of periodontal disease.

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Interleukin-10 is a key regulatory cytokine that has significant effects on both the innate and adaptive immune responses. It is produced by a variety of cells, including activated macrophages (1), dendritic cells, B cells and regulatory T cells (2,3). By stimulating B-cell immunity while at the same time suppressing both innate immunity and antigen-specific T-cell responses, in particular T helper 1-mediated responses (4), the role of interleukin-10 in human chronic infections is both complex and critical (4–6). Interleukin-10 and interleukin-10 homologues have been used in experimental and clinical trials as immunosuppressive agents to treat chronic inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and transplant rejection (7,8). However, by suppressing the innate and protective adaptive immune responses, interleukin-10 is also involved in the persistence of bacteria and in the maintenance of chronic infections.

As a result, interleukin-10 has been cited as being of major significance in the immunopathogenesis of chronic inflammatory diseases, such as periodontal

disease (9,10). Indeed, interleukin-10 may be critical in controlling the balance between T helper 1 and T helper 2 cells in chronic periodontitis, whereby an excess of interleukin-10 may shift the balance in favour of a T helper 2 response and progressive disease, whereas a deficiency may lead to increased interleukin-1 production and increased tissue destruction.

The interleukin-10 gene is now well characterized and the upstream promoter region contains a number of polymorphisms that define common genetic variants at this locus (11,12). Molecular genetic studies have defined a number of haplotypes, and emerging evidence suggests that these haplotypes may be associated with differing levels of interleukin-10 secretion *in vitro* and *in vivo* (12–15). In the interleukin-10 gene, single nucleotide polymorphisms at positions −1082 (G/A), −819 (C/T) and −592 (C/A) form conserved haplotypes (GCC, ATA and ACC), which relate to different levels of *in vitro* production of interleukin-10 (14,15). The single nucleotide polymorphisms and microsatellite polymorphisms in the interleukin-10 promoter region are linked, with distinct associations observed between single nucleotide polymorphism haplotypes and microsatellite repeat number, allowing some measure of comparison of the previous studies carried out on these various polymorphisms at this locus (12,13). Furthermore, a number of studies have shown that allelic variation in the interleukin-10 promoter is associated with a variety of diseases in which immune dysregulation contributes to the pathogenesis (11). The dinucleotide repeat polymorphism at position −1064 in the interleukin-10 promoter (interleukin-10.G) is particularly interesting as structural variation at this locus defines common haplotypes in the population that may be biologically important (12,13).

Two previous case-control studies, however, failed to find any evidence of an association between the interleukin-10. G microsatellite polymorphism and aggressive periodontitis (early onset) (16,17). Further cross-sectional studies failed to find evidence for genetic differences with respect to interleukin-10 genes between controls and patients with aggressive periodontitis or chronic periodontitis. The single nucleotide polymorphisms investigated in these studies included −1082, −819 and −592 (18), −597 and −824 (19), −627 and −1082 (20) and −1082 (21). Recently, however, cross-sectional studies in Swedish and Turkish populations revealed that allelic variants of the −1087 single nucleotide polymorphism (22) and −597 single nucleotide polymorphism (23) were associated with severe chronic periodontitis, whereas a Brazilian population (predominantly Caucasoid) showed an association with −819 and −592 single nucleotide polymorphisms and chronic periodontitis (24). These conflicting results may be attributed to the fact that cross-sectional studies may not always show an association in diseases of multifactorial aetiology. The manifestation of disease results from the interaction of genes with environmental factors, and the cohort design is best suited to study gene–environment interactions. In this regard, we showed previously that interleukin-1 gene polymorphisms, per se, were not associated with the progression of periodontal disease over a 5-year period. Rather, significant interactive effects were found between interleukin-1 genotype and age, smoking and the presence of *Porphyromonas gingivalis* on disease progression (25), clearly demonstrating the interaction among genetic, environmental and microbiological factors in the pathogenesis of periodontal disease in humans. Therefore, the purpose of the present prospective study was to investigate the possible association between interleukin-10 gene polymorphisms and environmental risk factors in the progression of periodontal disease in a general adult population.

Subjects and method

Subjects

Ethical clearance for this study was obtained from the relevant institutional ethics committee. Subjects were recruited from a cohort of 504 volunteers, participating in a prospective longitudinal study on the progression of periodontal disease in a general adult community, on the basis of availability and consent to undergo genetic testing. Signed, informed consent was obtained from the 252 subjects recruited into the study. Characteristics of the population and study design have been reported previously (25–29). Briefly, periodontal probing depths and relative attachment levels were measured at six sites per tooth using the Florida Probe® (Florida Probe Co., Gainesville, FL, USA). Subgingival plaque samples (12 per subject) were also collected and assayed for the presence of *P. gingivalis*, *Actinobacillus* (now *Aggregatibacter*) *actinomycetemcomitans* and *Prevotella intermedia*, using a previously standardized enzyme-linked immunosorbent assay (29). Examinations took place at baseline and at 12, 24, 36, 48 and 60 mo. Current smoking status was recorded. Peripheral blood samples were collected at either the 4- or 5-year examination time-points on filter paper discs (Whatman 3MM, Whatman International Ltd, Maidstone, UK), air dried and stored in sealed containers until analysis. Interleukin-1 genotype was determined by Interleukin Genetics (Flagstaff, AZ, USA), as previously described (25,30).

Extraction of DNA

An area of filter paper containing dried blood was cut into small pieces and DNA was extracted by the addition of 100 μL of 50 mM NaOH and heating to 100°C for 10 min in a water bath. DNA extracts were neutralized by the addition of 20 μL of 1 M Tris-HCl.

Analysis of interleukin-10 promoter single nucleotide polymorphisms

The interleukin-10 −592 and −1082 promoter single nucleotide polymorphisms were analysed using an induced heteroduplex method, as previously described (31). Polymerase chain reactions were performed in 22.5 μL volumes containing 0.1 U of *Taq* (Bioline, London, UK) 140 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, pH 8.8, 0.1% (v/v) Tween 20, 1.5 mM $MgCl_2$, 0.24 mM of

each dNTP, 0.5 mM of each primer and 2.5 μL of DNA extract. The amplification parameters for the interleukin-10 −592 single nucleotide polymorphism comprised an initial denaturation at 95°C for 5 min followed by 40 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 1 min, followed by a final extension of 72°C for 5 min. Amplification for the interleukin-10 −1082 single nucleotide polymorphism was identical, except that annealing was carried out at 59°C. Induced heteroduplex-generating oligonucleotides (31) were a kind gift of Dr J. Bidwell (University of Bristol, Bristol, UK). These were amplified for 30 cycles according to the same protocol as that used for the genomic DNA samples. Heteroduplexes for the amplified DNA fragments spanning each interleukin-10 single nucleotide polymorphism were generated by mixing 5 μL of the appropriate amplified induced heteroduplex-generating oligonucleotide with 20 μL of each amplified DNA sample, followed by denaturation at 95°C for 5 min and slow cooling from 95 to 37°C over 30 min. Heteroduplexes were resolved by polyacrylamide gel electrophoresis, as described above.

Haplotypes with respect to −1082 (G/A), −819 (C/T) and −592 (C/A) single nucleotide polymorphisms were inferred from −592 and −1082 genotype results and known haplotype frequencies. Thus, published haplotype frequency data identify three common haplotypes with respect to the single nucleotide polymorphism alleles at positions −1082, −819 and −592: these are GCC, ATA and ACC (12). Thus, the T allele at position −819 is linked to the A allele at position −592, and the C allele at position −819 is linked to the C allele at position −592; therefore, the identity of the single nucleotide polymorphism allele at position −819 can be inferred from the single nucleotide polymorphism allele at position −592 without the need for genotyping the single nucleotide polymorphism at this position. Of the 252 original subjects, eight had genotypes comprising haplotypes other than the three most common haplotypes and were not considered further in the analysis.

Statistical analysis

A generalized linear model was used to analyse the numbers of interproximal sites per subject with periodontal probing depths of ≥4 mm, as previously described (25,28). Covariates, additional to interleukin-10 single nucleotide polymorphism haplotypes, included in the model as possible confounding effects were: age, gender, smoking status, interleukin-1 genotype, examination number, response (number of periodontal probing depths ≥4 mm) at previous examination (to account for first-order ante-dependence), presence/absence of *P. gingivalis* at previous examination and number of days between current and previous examinations. The presence or absence of *A. actinomycetemcomitans* and *P. intermedia* was not included in the analysis as neither of these organisms was previously found to be associated with disease progression in this population (27). A significance level of 5% was used for testing the main effect of interleukin-10 haplotype.

Results

The mean age of the subjects was 42.3 years (standard deviation 10.1). A summary of the characteristics of this population, according to interleukin-10 genotype, is shown in Table 1. Of the 252 samples originally analysed, eight had genotypes comprising unusual haplotypes and therefore were not considered further in the analysis. The distribution of the various interleukin-10 genotypes in the study population is shown in Fig. 1. The frequency of individuals with an increase in the number of sites (by at least two) with periodontal probing depths ≥ 4 mm from baseline to 5 years, according to genotype, is shown in Fig. 2.

In the generalized linear model analysis there was a significant main

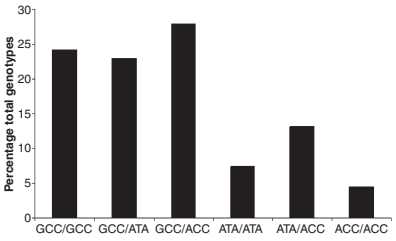

Fig. 1. Frequencies of interleukin-10 promoter genotypes in 244 individuals from a prospective longitudinal study on the progression of periodontal disease in a general adult community. Haplotypes with respect to −1082 (G/A), −819 (C/T) and −592 (C/A) single nucleotide polymorphisms were inferred from −592 and −1082 genotype results and published haplotype frequency data (12). Of the 252 samples originally analysed, eight had genotypes comprising unusual haplotypes and are not represented in this figure.

Table 1. Baseline characteristics of individuals in relation to interleukin-10 genotype

	GCC/GCC and GCC/ATA ($n = 115$)	GCC/ACC and ATA/ATA ($n = 86$)	ATA/ACC and ACC/ACC ($n = 43$)
Age (mean ± SD)	42.5 ± 9.9 years	42.6 ± 10.0 years	40.84 ± 11.3 years
Number (%) of males	65 (56.5%)	44 (51.2%)	21 (48.8%)
Number (%) of smokers	10 (8.7%)	7 (8.1%)	5 (11.6%)
Number (%) interleukin-1 genotype positive	48 (41.7%)	27 (31.4%)	18 (41.9%)
Number (%) *P. gingivalis* positive	16 (13.9%)	6 (7.0%)	4 (9.3%)
Number (%) with ≥1 site with periodontal probing depths ≥ 4 mm	67 (58.3%)	51 (59.3%)	30 (69.8%)

SD, standard deviation.

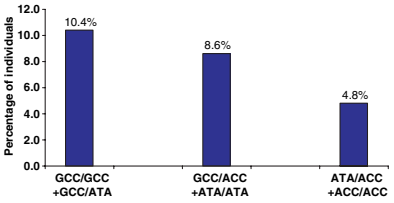

Fig. 2. Frequency of individuals with an increase (≥ 2) in the number of sites with periodontal probing depths of ≥ 4 mm from baseline to 5 years, according to interleukin-10 genotypes.

effect of interleukin-10 haplotypes, with patients having either the ATA/ACC or the ACC/ACC genotype showing a significantly (p-value ≈ 0.02) smaller response (in terms of numbers of periodontal probing depths ≥4 mm) than the other haplotypes. For individuals with other genotypes compared to those with the ATA/ACC or the ACC/ACC genotype, the odds of a periodontal probing depth being ≥4 mm were increased by an estimated factor of 1.25. Or, to put it another way, individuals with a relatively small number of periodontal pockets and a genotype other than ATA/ACC or ACC/ACC would be expected to have ≈25% more periodontal probing depths of ≥4 mm than those with ATA/ACC or ACC/ACC genotypes. This compares with a significant (p-value < 0.001) age effect estimate of an odds ratio of 1.015 per year, so that an individual without the ATA/ACC or the ACC/ACC genotype is equivalent, as far as mean number of periodontal probing depths of ≥4 mm is concerned, to individuals with the ATA/ACC or the ACC/ACC genotype, who are about 15 years older.

The effect of smoking was also significant (p-value < 0.001) and about twice the magnitude of the above interleukin-10 haplotype effect, with a smoker having about the same number of periodontal probing depths of ≥4 mm as a nonsmoker who was 27 years older.

Discussion

The present study has shown a significant relationship between interleukin-10 genotype and periodontal disease progression in this Australian population. Individuals with ATA/ACC or ACC/ACC genotypes had less disease progression than those with other genotypes. In this context, the ATA/ACC or ACC/ACC genotypes could be considered to be protective in those patients with disease progression such that disease progression is ameliorated. The contribution of the well-recognized risk factors – age and smoking – to disease progression is in addition to this effect. Individuals with genotypes other than these putative protective genotypes can be expected to have similar disease experience to individuals with the protective genotypes who are several years older. However, a smoker with the protective genotypes can expect to have more disease than a nonsmoker with the other genotypes.

While some studies have shown strong associations between interleukin-10 gene polymorphisms and diseases such as reactive arthritis (32) and primary Sjögren's syndrome (33), none, to date, has shown an association with aggressive periodontitis (16–20). The majority of studies looking at chronic periodontitis have also shown no association (18–21), with only three showing an association (22–24). The present study, however, has shown that certain interleukin-10 gene polymorphisms can be considered genetic risk factors for the progression of chronic periodontitis. The longitudinal study design enabled the relationship between interleukin-10 gene polymorphisms and incident disease progression to be studied. This is in contrast to the previous studies, referred to above, which were cross-sectional and therefore a reflection of past disease. Moreover, the present study was also able to take into consideration the contribution of other recognized risk factors, such as age, and the environmental factor, smoking.

Interleukin-10 is a major regulatory cytokine; on the one hand it inhibits the production of pro-inflammatory cytokines from polymorphonuclear neutrophils and macrophages (7) but up-regulates the recruitment and activation of B cells (5). It further down-regulates the T helper 1 response by inhibiting both interleukin-12 and interleukin-18 production (7). It is now generally accepted that chronic periodontitis is primarily a B-cell/plasma cell lesion (34) and while controversy still exists regarding the role of T helper 1 and T helper 2 cells in the control of this B-cell lesion (9,35), it is conceivable that interleukin-10 contributes significantly to maintaining the balance. We have presented evidence for a role of certain interleukin-10 promoter genotypes in slower progression of periodontal disease. It is significant that the genotypes in question (ATA/ACC and ACC/ACC) have been consistently associated with low levels of interleukin-10 production in *in vitro* assays (15,36,37). It is possible that subjects with other genotypes (e.g. those containing GCC and ATA haplotypes) have a relatively higher production of interleukin-10, which suppresses the innate immune response and hence the production of interleukin-12 and interleukin-18, thus favouring the development of a B-cell lesion associated with disease progression in periodontitis (35). In support of this hypothesis there is some evidence that the GCC and ATA haplotypes also influence interleukin-10 levels *in vitro* and *in vivo*, but in general terms the results have not been consistent (12,14,15,37–39).

Increased amounts of interleukin-10 are associated with a number of infections, including *Mycobacterium tuberculosis*, *Streptococcus pneumoniae and Pseudomonas aeruginosa* (6). The level of interleukin-10 is higher in lepromatous leprosy compared with tuberculoid leprosy, reflecting the shift towards a T helper 2-mediated response (40), but its role in a number of diseases is conflicting and in some instances increased amounts are associated with a shift towards a T helper 1 response (41). Clearly, the results of the present study suggest that subjects without the low production genotypes (ATA/ACC and ACC/ACC) may have increased progression of chronic periodontitis.

In contrast, other studies have shown that high levels of interleukin-10 may protect the host from excessive tissue destruction associated with autoimmunity and chronic inflamma-

tion (42). It is interesting to note that respiratory syncytial virus bronchiolitis appears to be associated with the ability to produce both high and low levels of interleukin-10 (43). Indeed, the same may be true for periodontal disease, where a lack of interleukin-10 may fail to protect the host from excess tissue damage while high levels suppress innate immunity and favour a shift to a T helper 2-mediated B-cell response.

The present study has demonstrated the additive effect of other risk factors, such as age and smoking, to that of the interleukin-10 polymorphism. We have previously shown that smoking substantially impairs the natural repair phases that occur between episodes of progression of periodontal disease, with smokers having only 28% of the healing capacity of nonsmokers, although the mechanisms involved are not understood (26). Similarly, we have previously shown that interleukin-1 genotype is associated with disease progression but only in combination with putative primary risk factors such as age, smoking and the presence of *P. gingivalis* (25,27). In the present study, there was a significant main effect of interleukin-10 haplotypes, such that individuals with the putative protective genotypes showed significantly less disease progression after allowing for the confounding effects of interleukin-1 and *P. gingivalis*. The fact that previous cross-sectional studies were unable to show an association between interleukin-10 polymorphism and various periodontal diseases may reflect the fine balance between interleukin-10 and disease expression and progression. The findings of the current study highlight the necessity for prospective longitudinal studies in elucidating the relative contributions of various factors in diseases with a multifactorial aetiology where there is interplay between genes and the environment.

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