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ABSTRACT

There is strong evidence that genetic as well as environmental factors affect the development of periodontitis, and some suggestion that aggressive and chronic forms of the disease share the same genetic predisposition. This study addresses the hypothesis that there are both shared and unique genetic associations in these forms of periodontitis. A sample of 51 patients with aggressive disease, 57 patients with chronic disease, and 100 healthy controls was recruited for this study. Ten functional polymorphisms in 7 candidate genes were genotyped. The results show statistically significant ($p \leq 0.05$) differences between genotype frequencies in aggressive and controls (IL-1B +3954 & IL-6 -174); chronic and controls (IL-6 -174 & VDR -1056); chronic and aggressive periodontitis (IL-1A -889); and periodontitis as a whole and controls (VDR -1056, TLR-4 399 & IL-6 -174). These results suggest that there are in fact both shared and unique genetic associations in aggressive and chronic periodontitis.

KEY WORDS: periodontitis, genetic susceptibility, polymorphisms, genes, cytokines.

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INTRODUCTION

There is strong evidence that genetic as well as environmental factors affect the age of onset, severity, and lifetime risk of developing periodontitis. The evidence for genetic susceptibility comes from studies in twins (Michalowicz *et al.*, 1991; Corey *et al.*, 1993), linkage studies and segregation analyses in families with aggressive (early onset) forms of periodontitis (Beaty *et al.*, 1987; Marazita *et al.*, 1994), and association studies (Kornman *et al.*, 1997; Suzuki *et al.*, 2004).

Previous studies have examined the association between polymorphisms in candidate genes and periodontitis. Since the attachment loss observed in periodontitis subjects has been shown to be induced by Gram-negative anaerobic bacteria, and mediated by inflammatory activation of endogenous matrix metalloproteinases, candidate genes have been selected from among pro-inflammatory and regulatory cytokines, the hypothesis being that increased or unregulated production of inflammatory cytokines (such as IL-1 and TNFα), in response to bacterial biofilms, occurs in subjects carrying functional polymorphisms in these genes. IL-1 polymorphisms have been associated with severity of periodontitis (Kornman *et al.*, 1997; McDevitt *et al.*, 2000), tooth loss (McGuire and Nunn, 1999), and increased bleeding on probing (Lang *et al.*, 2000). Several other candidate genes have also been examined, due to their association with periodontal pathology—for example, IL-10 for its anti-inflammatory activities (Kinane *et al.*, 1999; Yamazaki *et al.*, 2001), and vitamin D-receptor for its involvement in bone resorption and metabolism (Hennig *et al.*, 1999; Inagaki *et al.*, 2003).

Identification of genetic risk factors for periodontitis has been further complicated by the likely existence of multiple presentations of disease: chronic and aggressive periodontitis. Furthermore, a recent analysis of the available evidence seems to indicate that there is a degree of heterogeneity in genetic risk factors for periodontitis (Tonetti and Mombelli, 1999). To date, most studies have examined the association of a candidate polymorphism with only one form of periodontitis at a time. Recently, a group has examined 310 polymorphisms in a group of aggressive and chronic patients of Japanese ethnicity, and has showed differences in associations between the two forms of disease (Suzuki *et al.*, 2004). However, no investigation has attempted to assess if the phenotypic differences seen between aggressive and chronic periodontitis are reflected in a difference in associations with several polymorphisms in a Caucasian population.

The aim of this study was to assess if the odds of carriage of specific genotypes were significantly different in aggressive and/or chronic periodontitis patients with respect to the general population. For the purpose of this study, a variety of genotypes was selected for examination, based on the putative role of the gene product on the detection, amplification, and control of the inflammatory process and connective tissue metabolism. Functional polymorphisms in pattern recognition genes (toll-like receptor-4, TLR-4), pro-inflammatory cytokines (IL-1A, IL-1B, TNFα), regulatory cytokines (IL-6, IL-10), and a connective tissue metabolism-associated-gene (VDR) were determined in a population of Caucasian ethnicity.

MATERIALS & METHODS

Subjects

Patients with severe periodontal disease were recruited from the Department of Periodontology at the Eastman Dental Hospital. All subjects gave informed consent to participate in this study, which had been approved by the Eastman/University College London Hospitals joint ethics committee (Ref #98/024). The patients were diagnosed according to the criteria of the IWC 1999 International Workshop for a Classification of Periodontal Diseases and Conditions (Armitage, 1999), and were assigned as having either aggressive or chronic periodontal disease. Cases of uncertain clinical classification were excluded from the study. A detailed medical, oral, and family medical history was taken, followed by a complete periodontal examination that included six-point periodontal probing and appropriate radiographs. A clinical diagnosis of aggressive periodontal disease was made for 51 patients, while 57 patients were diagnosed as having chronic periodontal disease. All subjects included in this study were Caucasians, but of mixed smoking status, age, and gender. Of the 'aggressive' patients, 37 were female and 14 male, and of those diagnosed with chronic periodontal disease, 33 were female and 24 male. A Caucasian control group was used for this study, and consisted of 100 unrelated healthy individuals of unknown periodontal status, 56 female and 44 male, recruited from the local Blood Transfusion Service Unit. It is possible that this sample might contain a few individuals with minor periodontal disease; if so, this will have the effect of reducing the sensitivity of this study to detect genetic associations.

Table 1. The Primer Sequences, PCR Cycling Conditions, and Restriction Enzymes Used to Amplify Each Marker and Determine the Genotypes

Marker	Primer Sequences
IL-1 A (-889)	5′-AAGCTTGTTCTACCACCTGAACTAGGC-3′ 5′-TTACATATGAGCCTTCCATG-3′ 95°C, 4 min, 45 cycles; 94°C, 1 min; 50°C, 1 min; 72°C, 1 min *Nco 1* enzyme digest
IL1 B (-511)	5′TGGCATTGATCTGGTTCATC-3′ 5′-GTTTAGGAATCTTCCCACTT-3′ 95°C, 4 min, 35 cycles; 95°C, 1 min; 53°C, 1 min; 74°C, 1 min *Bco 1* enzyme digest
IL-1 B (+3954)	5′-CTCAGGTGTCCTCGAAGAAATCAAA-3′ 5′-GCTTTTTTGCTGTGAGTCCCG-3′ 95°C, 4 min, 35 cycles; 95°C, 1 min; 67.5°C, 1 min; 74°C, 1 min *Taq 1* enzyme digest
IL-6 (-174)	5′-TGACTTCAGCTTTACTCTTTGT-3′ 5′-CTGATTGGAAACCTTAT-3′ 95°C, 4 min, 35 cycles; 95°C, 45 sec; 63°C, 1 min; 72°C, 75 sec *Hsp 92* II enzyme digest
IL-10 (-627)	5′-CTTAGGTCACAGTGACGTGG-3′ 5′-GTGAGCACTACCTGACTAGC-3 95°C, 4 min, 35 cycles; 94°C, 1 min; 50°C, 1 min; 70°C, 1 min *Rsa 1* enzyme digest
IL-10 (-1082)	5′-TCT GAA GAA GTC CTG ATG TCA CTG-3′ 5′-ACT TTC ATC TTA CCT ATC CCT ACT TCC-3′ 40 cycles; 95°C, 1 min; 52°C, 1 min; 72°C, 1 min; 72°C, 1 min *Mnl I* enzyme digest
VDR (1056)	5′-CAGAGCATGGACAGGGAGCAAG-3′ 5′-GGATGTACGTCTGCAGTGTG-3′ 95°C, 4 min, 35 cycles; 95°C, 45 sec; 63°C, 1 min; 72°C, 75 sec *Taq 1* enzyme digest
TLR-4 (-299)	5′-GATTAGCATACTTAGACTACTACTACCTCCTCCATG-3′ 5′-GATCAACTTCTGAAAAAGCATTCCCAC-3′ 95°C, 4 min, 30 cycles; 95°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec *Nco 1* enzyme digest
TLR-4 (-399)	5′-GGT TGC TGT TCT CAA AGT GAT TTT GGG AGA A-3′ 5′-ACC TGA AGA CTG GAG GAG TGA GTT AAA TGC T-3′ 95°C, 4 min, 30 cycles; 95°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec *HinfI* enzyme digest
TNFα (-308)	5′-AGG CAA TAG GTT TTG AGG GCC AT-3′ 5′-TCC TCC CTG CTC CGA TTC CG-3′ 94°C, 4 min, 35 cycles; 94°C, 2 min; 60°C, 1 min; 72°C, 1 min *Taq 1* enzyme digest

Sample Collection and DNA Extraction

From each patient, we collected 10 mL of blood by venipuncture in the ante-cubital fossa. The blood samples were collected in sodium EDTA vacutainers and stored at -20°C. The extraction of DNA was performed with the Nucleon® kit following the manufacturer's protocol.

Each sample of DNA was analyzed for polymorphisms in the IL-1A gene at position -889 (McDowell *et al.*, 1995), in the IL-1B gene at positions -511 (Di Giovine *et al.*, 1992) and +3953 (Pociot *et al.*, 1992), in the promoter region of the IL-6 gene at position -174 (Fernandez-Real *et al.*, 2001), in the IL-10 gene at positions -627 and -1082 (Aithal *et al.*, 2001), in the Taq I polymorphism of the vitamin D receptor gene (VDR) (Hennig *et al.*, 1999), in the TNFα gene at position -308 (Kornman *et al.*, 1997), and in the TLR-4 gene at positions -299 and -399 (Lorenz *et al.*, 2001). Primer sequences and conditions are shown for each primer pair (Table 1).

Genotype Determination

DNA samples were amplified by PCR with specific primers for each examined polymorphism (Table 1). The PCR reaction was performed in a final volume of 50 μL in a buffer containing 75 mM Tris-HCl, 20 mM $(NH_4)_2$ SO_4, 0.2 mM each dNTP, 0.01% Tween 20, 1.5-2.5 mM $MgCl_2$ (depending on marker), 0.4 μL Taq DNA polymerase, and 20 ρM of each primer. After cycling, the whole 50-μL PCR product was digested with the appropriate restriction endonuclease (Table 1). The alleles were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. Following electrophoresis, the PCR products were visualized under ultraviolet light. The images obtained

were stored digitally for later analysis.

Statistical Methods

To determine whether any significant differences in polymorphism frequencies occurred between the case and control populations, and between the different forms of periodontitis, we compared allele and genotype frequencies, using the chi-square method. Where significant p-values were generated, the odds ratio was calculated (Bland and Altman, 2000). We performed illustrative power calculations for aggressive, chronic, and aggressive/chronic to estimate the relevance of the p-values produced from this dataset; methodology for discrete traits in case-control studies was utilized (Purcell *et al.*, 2003). Periodontitis prevalence was assumed to be 0.013 for aggressive (Albandar *et al.*, 1997), 0.05 for chronic (Kelly *et al.*, 2000), and 0.063 for aggressive/chronic. The level of clinical relevance for major-effect genes was defined as a heterozygous odds ratio of 3 and a homozygous risk of 5, assuming a risk allele frequency of 10%.

RESULTS

The power calculations performed for this study show that the sample size required to ascertain the significance of association of periodontal disease to the studied genetic polymorphisms with an alpha value of 0.05 and 80% power was 47 for aggressive, 43 for chronic, and 63 patients for aggressive/chronic groups combined. This shows that our sample

Table 2. Genotype Data for the 10 Markers Examined in the Aggressive (N = 51), Chronic (N = 57), and All Periodontitis (N = 108) Groups and 100 Healthy Controls and the Results of the CLUMP Analysis of the Genotype Distribution for Each Marker

Gene Polymorphisms	Genotypes	Control (Con), N (%)	Aggressive (AGP), N (%)	Chronic (CP), N (%)	Periodontitis (Perio), N (%)
IL-1A -889	1,1	44 (44%)	31 (62%)	16 (28%)	47 (44%)
	1,2	37 (37%)	11 (22%)	26 (46%)	37 (35%)
	2,2	19 (19%)	8 (16%)	15 (26%)	23 (21%)
Chi-squared analysis		AGP *vs.* Con	CP *vs.* Con	AGP *vs.* CP	Perio *vs.* Con
		0.09	0.36	0.001**	0.88
		Odds ratio calculated for aggressive *vs.* chronic (1,1 *vs.* 1,2 + 2,2) = 4.2 (95% CI 1.9-9.4)			
IL-1B +3954	1,1	58 (59%)	28 (57%)	32 (58%)	60 (58%)
	1,2	32 (33%)	9 (18%)	17 (31%)	26 (25%)
	2,2	8 (8%)	12 (24%)	6 (11%)	18 (17%)
Chi-squared analysis		AGP *vs.* Con	CP *vs.* Con	AGP *vs.* CP	Perio *vs.* Con
		0.012*	0.848	0.111	0.111
		Odds ratio calculated for aggressive *vs.* control (2,2 *vs.* 1,1 + 1,2) = 3.6 (95% CI 1.4-9.7)			
IL-1B -511	1,1	51 (51%)	23 (45%)	27 (47%)	50 (47%)
	1,2	39 (39%)	21 (41%)	26 (46%)	47 (44%)
	2,2	10 (10%)	6 (12%)	4 (7%)	10 (9%)
Chi-squared analysis		AGP *vs.* Con	CP *vs.* Con	AGP *vs.* CP	Perio *vs.* Con
		0.83	0.65	0.67	0.77
TNFα -308	1,1	58 (60%)	26 (52%)	36 (65%)	62 (59%)
	1,2	39 (40%)	24 (48%)	18 (33%)	42 (40%)
	2,2	0 (0%)	0 (0%)	1 (2%)	1 (1%)
Chi-squared analysis		AGP *vs.* Con	CP *vs.* Con	AGP *vs.* CP	Perio *vs.* Con
		0.66	0.29	0.19	0.62
TLR-4 -299	1,1	90 (93%)	37 (82%)	47 (89%)	84 (86%)
	1,2	7 (7%)	8 (18%)	6 (11%)	14 (14%)
	2,2	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Chi-squared analysis		AGP *vs.* Con	CP *vs.* Con	AGP *vs.* CP	Perio *vs.* Con
		0.16	0.69	0.66	0.30
TLR-4 -399	1,1	78 (82%)	46 (94%)	50 (93%)	96 (93%)
	1,2	17 (18%)	3 (6%)	4 (7%)	7 (7%)
	2,2	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Chi-squared analysis		AGP *vs.* Con	CP *vs.* Con	AGP *vs.* CP	Perio *vs.* Con
		0.08	0.095	0.10	0.002**
		Odds ratio calculated for periodontitis *vs.* control (1,1 *vs.* 1,2 + 2,2) = 3.0 (95% CI 1.2-7.6)			
IL-6 -174	G,G	55 (55.5%)	30 (61.0%)	22 (38.6%)	52 (49%)
	G,C	19 (19.2%)	13 (26.5%)	24 (42%)	37 (35%)
	C,C	25 (25.3%)	6 (12.5%)	11 (19.4%)	17 (16%)
Chi-squared analysis		AGP *vs.* Con	CP *vs.* Con	AGP *vs.* CP	Perio *vs.* Con
		0.16	0.008**	0.07	0.02*
		Odds ratio calculated for chronic *vs.* control (G,G + G,C *vs.* CC) = 3.1 (95% CI 1.5-6.3)			
IL-10 -627	C,C	58 (60%)	28 (62%)	38 (68%)	66 (65%)
	C,A	35 (36%)	15 (33%)	15 (27%)	30 (30%)
	A,A	4 (4%)	2 (4%)	3 (5%)	5 (5%)
Chi-squared analysis		AGP *vs.* Con	CP *vs.* Con	AGP *vs.* CP	Perio *vs.* Con
		0.949	0.49	0.76	0.62
IL-10 -1082	G,G	17 (18%)	15 (31%)	13 (24%)	28 (27%)
	G,A	47 (51%)	17 (35%)	24 (43%)	41 (40%)
	A,A	28 (31%)	16 (34%)	18 (33%)	34 (33%)
Chi-squared analysis		AGP *vs.* Con	CP *vs.* Con	AGP *vs.* CP	Perio *vs.* Con
		0.15	0.636	0.61	0.2
VDR 1056	T,T	27 (28%)	19 (39%)	28 (51%)	47 (45%)
	T,t	56 (57%)	24 (49%)	19 (35%)	43 (41%)
	t,t	15 (15%)	6 (12%)	8 (14%)	14 (14%)
Chi-squared analysis		AGP *vs.* Con	CP *vs.* Con	AGP *vs.* CP	Perio *vs.* Con
		0.38	0.01*	0.32	0.02*
		Odds ratio calculated for chronic *vs.* control (T,T *vs.* T,t + t,t) = 2.7 (95% CI 1.4-5.4)			

size was large enough to detect association with an acceptable level of confidence.

The genotype data gathered for all 10 markers for the patient and control groups are shown (Table 2). The allele and genotype distributions observed in the control population were similar to those previously reported in Caucasian populations. The association data were analyzed by CLUMP, and the odds ratios and 95% confidence intervals for any significant result were calculated as described; the results are shown (Table 2). Analyses were performed for four comparisons: aggressive *vs.* control, chronic *vs.* control, aggressive *vs.* chronic, and combining periodontal disease in total (aggressive/chronic) *vs.* control. There were 7 significant findings: 1 for aggressive *vs.* control, 2 for chronic *vs.* control, 3 for aggressive/chronic *vs.* control, and 1 for aggressive *vs.* chronic.

No statistically significant differences ($P > 0.05$) in genotype and allelic distribution for the IL-1A (-889) polymorphism were detected between aggressive and controls, chronic and controls, and aggressive/chronic and controls. The only statistically significant difference was between aggressive and chronic for genotype (T_1, $p = 0.001$) and allelic distributions (T_1, $p = 0.008$). Chronic patients had significantly increased odds of carrying the allele 2 genotype than did aggressive subjects [odds ratio = 4.2 (95% CI 1.9 to 9.4)].

A statistically significant difference in genotypic frequencies between aggressive subjects and controls (T_1, $p = 0.012$) was observed for the IL-1B (+3954) gene polymorphism. The 2,2 genotype was associated with an increased susceptibility to aggressive periodontitis [odds ratio = 3.6 (95% CI 1.4 to 9.7)]. No significant differences in allelic distributions were observed for this marker.

With regard to the IL-6 (-174) gene polymorphism, statistically significant differences in genotypic distribution were observed between subjects with chronic periodontitis and controls (T_1, $p = 0.008$) and between aggressive/chronic subjects and controls (T_1, $p = 0.02$). The GC genotype was overrepresented in chronic periodontitis subjects (42% in chronic compared with 19.2% in controls and 26.5% in aggressive), suggesting that the carriage of the GC genotype was associated with an increased risk of chronic disease [odds ratio = 3.1 (95% CI 1.5 to 6.3)].

The VDR (+1056) gene polymorphism displayed statistically significant differences in allele (T_1, $p = 0.03$) and genotype (T_1, $p = 0.01$) distribution in comparisons of the chronic and control groups. Similarly, for periodontal disease in total (aggressive/chronic) and controls, there were statistically significant differences for allele (T_1, $p = 0.04$) and genotype (T_1, $p = 0.02$) distributions. The Tt and tt genotypes were more prevalent in controls compared with patients with chronic disease [odds ratio = 2.7 (95% CI 1.4 to 5.4)].

For the TLR-4 (-399) gene polymorphism, the only statistically significant difference was between total subjects with periodontal disease (aggressive/chronic) and controls. This was in both allele (T_1, $p = 0.033$) and genotype (T_1, $p = 0.002$) distributions, with allele 1 and 1,1 genotypes being more frequent in periodontitis subjects than in controls [odds ratio = 3.0 (95% CI 1.2 to 7.6)].

No statistically significant differences were found for the 5 other markers examined (IL-1B, -511; IL-10, -627 and -1082; TNFα, -308; and TLR-4, -299).

Kornman *et al.* (1997) have described a "composite genotype" that consisted of at least 1 allele 2 from both IL-1A -889 and IL-1B +3954 determining the severity of chronic periodontal disease. Therefore, the frequency of this "composite genotype" in aggressive and chronic subjects was analyzed. We found no significant difference in "composite genotypic" distribution between chronic subjects and controls. The analysis did show that there was a statistically significant difference between subjects with aggressive periodontitis and controls (T_1, $p = 0.014$), and aggressive and chronic subjects (T_1, $p = 0.01$), with "composite genotype"-negative patients more prevalent in the aggressive group than either chronic [odds ratio = 3.0 (95% CI 1.2 to 7.6)] or controls [odds ratio = 2.9 (95% CI 1.2-6.7)].

DISCUSSION

All of the genes screened in this study play a role in inflammation and immunity, and as such, might play a role in the altered inflammatory responses leading to the development of the symptoms seen in periodontal disease. The polymorphisms examined are all functional and have effects on either the amount of protein produced or the activity of the protein produced from each gene. Coupled with the involvement of the genes in immunity, this makes them good candidates for this type of genetic association study.

The power calculation, while making many assumptions, shows that the sample size used in this study is sufficient to detect most associations, but might be considered to be marginal in a search for associations to genes with relatively small effects. So caution must be exercised when one draws any conclusions about the marginal negative results seen. For instance, examination of the TLR-4 -399 results shows a statistically significant result for all periodontal disease (odds ratio = 3.0 (95% CI 1.2-7.6), but not for aggressive or chronic disease alone. This could be a reflection of the minor effect of the TLR-4 polymorphism on disease susceptibility. If larger samples of aggressive and chronic patients were used, and if a "disease free" control group were used, significant associations for either or both might have been observed. Alternatively, the very rare nature of homozygous 2,2 individuals may be responsible. This result does suggest, however, that the innate immunity, in which TLR-4 is involved, does play a role in one's development of, or susceptibility to, periodontal disease.

The polymorphisms that produced significant p-values in this study had different functional effects on the proteins produced. The IL-1A -889 gene polymorphism allele 2 was associated with a four-fold increase in levels of IL-1α in gingival crevicular fluid (Shirodaria *et al.*, 2000); the IL-1B +3954 gene polymorphism was associated with variations in monocyte production and the production of IL-1β by monocytes during inflammation (Pociot *et al.*, 1992); the IL-6 -174 gene polymorphism has previously been found to be related to different transcription rates and plasma levels of IL-6 in healthy individuals (Fernandez-Real *et al.*, 2001); and the VDR 1056 gene polymorphism may affect the degree of inhibition of the production of IL-1α and IL-6 by the VDR (Morrison, 1998).

Hennig *et al.* (1999) analyzed the genotypes of 69 patients with early-onset periodontitis, including 20 patients with localized disease and 72 controls, and concluded that the carriage of the less-frequent t-allele in VDR was associated with an increased risk of developing localized, but not generalized, disease. However, our

results contradicted these findings and suggested that the t-allele may be protective against periodontal diseases. One explanation of this difference is that the presence of the t-allele in cases of localized disease may limit the extent of periodontitis, thus preventing the development of generalized disease and strengthening the view that these genes modify the phenotype produced by the underlying susceptibility to periodontal disease. Another piece of evidence pointing to this modification of phenotype is the statistically significant underrepresentation of "composite genotype"-positive (Kornman *et al.*, 1997) patients in the aggressive group compared with both the control and the chronic groups. This result suggests that if the underlying genetic susceptibility is the same for aggressive and chronic periodontal disease, then the presence or absence of the genotype-positive status may determine the phenotypic manifestation of this susceptibility. Therefore, these results suggest that if there is a shared genetic etiology in the periodontal diseases, then the phenotypic variations seen are due to the modification of this susceptibility by many different gene variations.

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