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Analysis of TNF-α (-308) polymorphism and gingival crevicular fluid TNF-α levels in aggressive and chronic periodontitis: A preliminary report

Özlem Özer Yücel [a,*], Ezel Berker [b], Lütfiye Mesci [c], Kenan Eratalay [b], Eser Tepe [d], İlhan Tezcan [e]

[a] Department of Oral Pathology, Faculty of Dentistry, University of Gazi, Ankara, Turkey
[b] Department of Periodontology, Faculty of Dentistry, Hacettepe University, Ankara, Turkey
[c] Genetic Technologies, Diagnostics and Therapies INC., İstanbul, Turkey
[d] T.C. Ministry of Health, Tepebaşı Mouth and Tooth Health Hospital, Ankara, Turkey
[e] Hacettepe University, Medical Faculty, Children's Hospital, Immunology Unit, Ankara, Turkey

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ABSTRACT

Objectives: The purpose of this study was to determine the differences in the distribution of TNF-α (-308) gene polymorphism among aggressive periodontitis, chronic periodontitis and periodontally healthy individuals and also to investigate whether this polymorphism is associated with gingival crevicular fluid TNF-α levels and periodontal disease severity.

Material and methods: A total of 93 individuals were enrolled in the study including 38 aggressive periodontitis, 29 chronic periodontitis patients, and 26 healthy controls. Single nucleotide polymorphism at TNF-α (-308) is analyzed by PCR-RFLP method. Gingival crevicular fluid samples were analyzed for TNF-α, using ELISA.

Results: The distribution of genotypes and allele frequencies for TNF-α (-308) were similar among the groups. After stratification of patients with respect to attachment level, aggressive periodontitis patients with clinical attachment level ⩾4 mm was observed to have a higher frequency of TNF-α (-308) allele 2 compared to the chronic periodontitis patients with clinical attachment level ⩾4 mm. No significant differences were found between the TNF-α levels of the different genotypes in spite of an insignificant increase in patient groups carrying TNF-α (-308) allele 2.

Conclusion: The results of this study revealed an association between TNF-α (-308) allele 2 frequency and aggressive periodontitis patients with clinical attachment level ⩾4 mm in the population studied.

1. Introduction

Periodontal diseases are chronic inflammatory infectious diseases that lead to the destruction of the tooth supporting tissues. While the presence of periodontopathogens is essential for the development of periodontal disease, host-related factors are the determining parameters for the progression and severity of the clinical outcome. Host response to bacteria triggers the secretion of pro-inflammatory mediators such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) leading extracellular matrix catabolism and bone resorption in periodontitis [1–3].

TNF-α is an important mediator in inflammatory reactions and appears to play a central role in the pathogenesis of severe chronic inflammatory diseases, autoimmune diseases and septic shock. Monocytes and macrophages are the potent cell types producing TNF-α. The local cellular effects of TNF-α include the adhesion of polymorphonuclear leukocytes (PMNs) to endothelial cells, degranulation of PMNs, activation of phagocytosis and intercellular adhesion molecule-1 (ICAM-1) expression. It is known that TNF-α induces bone and cartilage resorption by activating the osteoclasts in a similar way with IL-1 [4]. The amount of TNF-α was demonstrated at high levels in GCF and diseased periodontal tissues [5–7] and experimental studies have shown a central role for TNF-α in alveolar bone resorption [8,9].

Variation in cytokine levels among periodontitis patients is well documented and is associated with disease severity [10,11]. The mechanisms which regulate the production and biological activity of TNF-α are under genetic control and play an important role in

* Corresponding author at: Department of Oral Pathology, Faculty of Dentistry, Gazi University, 06510 Ankara, Turkey. Tel.: +90 3122034383; fax: +90 3122239226.
E-mail address: ozlemozertr@gmail.com (Ö. Özer Yücel).

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the initiation and progression of periodontal disease. Single nucleotide polymorphisms (SNPs), particularly the G to A transition at position – 308, have been demonstrated to increase TNF-α production up to fivefold in vitro [12]. Although the functional role of TNF-α (-308) polymorphism is shown in vitro, there are no studies demonstrating significant association between in vivo TNF levels and the assessed polymorphism in patients with periodontal disease. Limited number of studies reporting on the TNF-α (-308) polymorphism and TNF levels evaluated small numbers of subjects with chronic periodontitis and healthy individuals [13–15]. (Galbraith et al. 1999 [20 patients], Engebretson et al. 1999 [24 patients], Soga et. al. 2003 [15 healthy donors were used for monocyte cultures]) All the studies mentioned above, except by Engebretson et. al. [13] evaluated TNF expression in blood cultures [14–16]. In fact, the influence of different TNF-α genotypes on the phenotypic cytokine production is not fully established. The possible effect of TNF-α (-308) polymorphism on the periodontal disease severity and clinical outcome remains to be elucidated. While there is a considerable number of studies investigating the association of TNF-α (-308) gene polymorphisms in different ethnicities [14,16–24], the literature contains no data regarding the association of TNF-α (-308) genotype, GCF TNF-α levels and clinical parameters in periodontal disease, concerning both aggressive and chronic periodontitis. Therefore, the aims of the present study were to analyze TNF-α (-308) genotype and allele frequency in both chronic and aggressive periodontitis patients, and also to investigate whether this polymorphism is associated with GCF TNF-α levels, periodontal disease severity and clinical parameters.

2. Materials and methods

2.1. Selection of subjects

Ninety-three adult Turkish subjects referred to the Department of Periodontics at Hacettepe University were included for the study. Informed consent was obtained from the patients and the protocol was approved by the Ethics Committee of Hacettepe University. The Turkish ethnicity belongs to the Caucasian racial group, and the study population was drawn from a homogenous group residing in the same geographic region.

The patients were diagnosed according to clinical and radiographic criteria as aggressive periodontitis (AP, $n = 38$) chronic periodontitis (CP, $n = 29$), and controls (C, $n = 26$). All of the subjects were non-smokers, or had a pack year history of less than or equal to 5, and had at least 20 teeth. Both chronic and aggressive periodontitis patients were classified according to the AAP (1999) classification [25]. Patients with CP had moderate-to-advanced periodontitis (probing depth $\geqslant$5 mm at least on six sites one of which is the anterior, bone loss $\geqslant$ 25%) and had not received any periodontal treatment before the time of examination. For a diagnosis of AP, subjects had to present with a generalized pattern of severe periodontal destruction (probing depth $\geqslant$5 mm at least on three sites other than first molars and incisors). The patients with AP had a mean age of 29.6 and were drawn from a sample of patients with a history of rapid attachment loss and bone destruction. C group subjects were periodontally healthy and had no proximal bone and attachment loss. None of the subjects had any known systemic disorders or used antibiotics and anti-inflammatory medications in the last 3 months. Subjects with active infectious diseases such as hepatitis, HIV infection, and tuberculosis or chronically treated with medications (phenytoin, cyclosporine-A, or calcium channel blockers), as well as females, who were lactating or pregnant, were excluded.

Clinical parameters included probing depth (PD), clinical attachment level (CAL), plaque index (PI) [26], gingival index (GI) [27] and bleeding on probing (BOP). Six sites were examined for each tooth: mesiobuccal, buccal, distobuccal, mesiolingual, lingual and distolingual. All the measurements were made by the same examiner.

2.2. Genotyping methods

Peripheral venous blood was obtained by standard venipuncture and genomic DNA was extracted using a DNA isolation kit (QIAGEN DNA mini kit). Polymerase chain reaction (PCR) analysis was performed following DNA extraction as described previously [16]. Details of the PCR-RFLP method is summarized below:

Forward primer: 5′-AGGCAATAGG-TTTTGAGGGCCAT. Reverse primer: 5′-TCCT-CCCTGCTCCGATTCCG-3′. Cycling was carried out for 1 cycle at 94 °C for 3 min, 35 cycles for 1 min each at 94 °C, 60 °C and 72 °C; and 1 cycle at 72 °C for 1 min. Digestion of PCR products with *NcoI* yielded 87 + 20-bp fragments (allele 1: G nucleotide at position -308), and a single 170-bp fragment (allele 2: A nucleotide at position -308). The restriction fragments were determined on 3% agarose gel electrophoresis, stained with ethidium bromide (DNA Grade + Nuisieve agarose, Rockland, ME, USA). The identification number of SNP was rs1800629, typically called -308.

2.3. GCF sampling

A total of 45 patients including 18 aggressive periodontitis, 17 chronic periodontitis, and 10 healthy subjects were sampled for the analysis of TNF-α in GCF. The selected sites included anterior maxillary sites with probing depth $\geqslant$5 mm and radiographic evidence of bone loss. The sampling teeth selected were free of restorations. GCF samples were collected from 6 maxillary sites per patient. The strips obtained from each patient were studied as pooled sample. Sampling teeth were isolated with cotton rolls and gently dried. A standard paper strip (Perio-paper, IDE Interstate, Amityville, NY, USA) was inserted into the sulcus to the depth of 1–2 mm for 30 s and the strips were moved immediately to a calibrated Periotron 8000 (Oroflow, Inc, NY, USA) to determine the GCF volume. Strips contaminated by blood were excluded from the sampled group. After collection of the gingival fluid, the strips were immediately placed in sterile Eppendorf tubes containing 10 mm NaH_2PO_4 and 150 mm NaCl, pH 7.2, followed by mixing and centrifugation at 800 g. The GCF samples were stored at −80 °C until subsequent analysis.

2.4. Assay of TNF-α

GCF samples were analyzed for TNF-α using commercially available enzyme-linked immunosorbent assay (ELISA, R&D System Inc., MN, USA). Analyses were performed according to the manufacturer's protocol. All ELISA determinations were performed in duplicate. Results were calculated using the standard curves created in each assay. GCF TNF-α levels were reported as total amount in picograms (pg), or as concentration (pg/μl) by converting Periotron units to microliters using calibration curves described previously [28].

2.5. Statistical analysis

The associations of allele and genotype frequencies in patient and control groups were analyzed using the χ^2 (Pearson ki-square, likelihood ratio) and Fisher's Exact test. The difference between the clinical parameters was determined by ANOVA test. Because the data were not normally distributed, the total amounts of cytokines in GCF were expressed as medians and the significance of differences was assessed using Kruskall–Wallis test. A p value of less than 0.05 was considered to be statistically significant. The Kruskall–Wallis