Abstract

Objectives: Nuclear factor kappa B (NF-κB) is a member of the transcription factor family, and it plays a key role in coordinating the expression of genes in many chronic inflammatory diseases. This study investigated the cytoplasmic and nuclear activation of NF-κB and the cytoplasmic expression of inhibitor kappa B (IkB) in gingival tissues of subjects who had chronic periodontitis.

Methods: Thirty-five patients were included in this study; 17 patients had chronic periodontitis, and 18 were healthy. Gingival tissues were obtained from each individual and then stained immunohistochemically. The obtained sections were examined under a stereomicroscope, and the numerical density values of the stained cells were computed using the stereologic method. A one-way analysis of variance (ANOVA) and a multiple range least significant difference (LSD) were used for intergroup comparisons (P=0.05).

Results: According to the immunohistochemical analysis of the cytoplasmic positive cells stained with IkB, statistically significant differences were found between the case and control groups. When comparing the cytoplasmic and nuclear positive immunoreactivity of p50 and p65, statistically significant differences were found between the diseased and control groups. Statistically significant correlations were also found between the clinical periodontal scores and the immunohistochemical results of the diseased subjects.

Conclusions: It was shown that NF-κB was highly activated in subjects who had chronic periodontitis, compared to healthy controls. The findings of this study can be useful in planning new treatment strategies for periodontal diseases. Further investigations are needed to understand more about the signaling mechanisms of inflammatory mediators and their interactions with NF-κB in chronic periodontitis. (Eur J Dent 2010;4:454-461)

Key words: Nuclear factor kappa B; Chronic periodontitis; Cytokine.
INTRODUCTION

Periodontitis is a chronic inflammatory disease caused by microbial dental plaque. Bacterial colonization and subsequent bacterial invasion into the gingival sulcus are the primary etiologic factors for periodontal diseases. In addition to pathogenic microorganisms in the biofilm, genetic and environmental factors (especially tobacco use) contribute to the cause of these diseases. Chronic periodontitis is the most common form of the periodontal diseases that result in loss of connective tissue attachment and bone support. Accordingly, chronic periodontitis is a major cause of tooth loss in adults. The interactions between microorganisms and host response play a key role in the disease’s pathogenesis. The immune and inflammatory responses to the chronic presence of microorganisms result in the destruction of structural components in the periodontium. Although bacteria are necessary for periodontal disease’s occurrence, a susceptible host is also needed. The host response is essentially protective, but both hypo-responsiveness and hyper-responsiveness in the immune system can result in enhanced tissue destruction. As with other chronic inflammatory diseases, the exact etiology of periodontitis is still obscure, and little is known about the molecular changes and the signaling cascade of the host response. Consequently, recent studies have endeavored to elucidate the role of host response in pathogenesis rather than examining the direct destruction of the tissues by microorganisms.

The pathogenesis of chronic inflammatory periodontal disease is likely modulated by genetic and environmental factors. Transcription factors are gene-specific factors that often act as links between genetic and environmental factors. Nuclear factor kappa B (NF-κB) is a transcription factor, first identified by Sen and Baltimore, that coordinates the expression of a wide variety of genes responsible for inflammation. NF-κB is an important family of transcription factors, and it represents a group of structurally related and evolutionarily conserved dimeric proteins that belong to the Rel family. Mammals express 5 NF-κB proteins, including NF-κB-1 (p50), NF-κB-2 (p52), Rel A (p65), Rel-B, and c-Rel. These proteins have structurally conserved amino-terminal 300-amino-acid regions, which contain the dimerization, nuclear-localization, and DNA-binding domains. The primary activated form of NF-κB is a heterodimer of the p65 subunit associated with either the p50 or the p52 subunit. In unstimulated cells, NF-κB is bound to its inhibitors, called inhibitor kappa B (IκB) proteins, and it resides in the cytoplasm as an inactive NF-κB/IκB complex. NF-κB can be activated within minutes by a variety of stimuli, such as tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), IL-6, reactive oxygen intermediates (ROIs), receptor activator NF-κB ligand, ultraviolet irradiation, lipopolysaccharides (LPS), and other bacterial and viral agents. Among them, the strongest activators of NF-κB are TNFα and IL-1β of the proinflammatory cytokines. NF-κB activation is regulated by direct interactions with IκB family members, including IκB-α, IκB-β, and IκB-ε. IκBs mask the nuclear localization sequences of NF-κB and hinder the recognition of the NF-κB nuclear localization signal (NLS) by the nuclear import machinery, thus retaining NF-κB in the cytoplasm. Following the stimulation of cells, IκB is first phosphorylated by IκB kinases (IKK) and then rapidly degraded by proteasomes. IκB degradation results in the translocation of NF-κB into the nucleus, where it binds to DNA regulatory sites (called κB sites) to activate specific target gene expression.

NF-κB activation by nuclear translocation plays a central role in inflammation through its ability to induce the transcription of proinflammatory genes. Cellular events associated with NF-κB activation include cell-to-cell adhesion, cell recruitment or transmigration of inflammatory cells, and the amplification or spreading of primary pathogenic signals. NF-κB activation is associated with many chronic immune and inflammatory diseases such as inflammatory bowel disease, asthma, oral lichen planus, pancreatitis, AIDS, and arthritis. However, several systemic conditions that are linked with periodontitis (such as atherosclerosis, diabetes mellitus, and smoking) are also associated with NF-κB activation. Stimulators that are identified as crucial NF-κB activators (such as LPS, TNFα, IL-1β, and RANKL) are found in abundance in the periodontal tissues of subjects who have periodontitis. These molecules are mentioned as important inducers of immune and inflammatory responses associated with soft tissue destruction and bone resorption in the tooth-supporting structures.
NF-κB activation in chronic periodontitis

Inflammation, the target genes of NF-κB in the inflammatory process are believed to facilitate the progression of periodontitis. Therefore, the present study compared the nuclear expression of activated NF-κB (p50/p65) and IκB in the gingival tissues of the subjects who had chronic periodontitis and the clinically healthy controls.

MATERIALS AND METHODS

Population selection

The study population was selected from patients who reported to the outpatient division of Atatürk University’s Faculty of Dentistry, Department of Periodontology, in Erzurum, Turkey, in 2007. The case and control groups comprised a total of 35 patients (17 chronic periodontitis and 18 healthy subjects). The groups excluded patients with any form of systemic disease, those who had taken any form of medication over the last six months, and smokers. The case group included 9 males and 8 females (29 to 46 years old; mean 36.7 years) who required tooth extraction due to poor prognosis. Eight males and 10 females (23 to 37 years old; mean 30.9 years) who required tooth extraction for reasons other than periodontal disease were selected for the control group. Periodontal conditions were diagnosed by radiographic and detailed periodontal examination, which included gingival inflammation assessment by bleeding on probing (BOP), probing depth (PD), plaque index (PI), calculus index (CI), mobility, and clinical attachment loss (CAL).

Collection of the tissues

The patients in the case and control groups were informed about the study so that they could give their consent. 1 to 2 mm of gingival tissue was taken from the buccal side of the tooth of each subject before extraction. Next, the biopsies were immediately fixed in 10% neutral buffered formalin and sent to the pathology department for immunohistochemistry and stereologic examination.

Immunohistochemistry

The samples were dehydrated in a graded ethanol series and embedded in paraffin wax for immunohistochemical analysis. Sections approximately 10 μm thick were taken from paraffin blocks to positive charged lamds and were incubated by 15-minute segments at 70°C. All the sections were deparaffinized in graded xylool, then hydrated in different absolute (99.8%) alcohol solutions, and then rinsed in distilled water. After the antigen retrieval procedure, using a 0.01 M citrate buffer, the sections were rinsed in distilled water and then incubated with 3% H₂O₂. The sections were rinsed with phosphate-buffered saline (PBS/pH = 7.4). Sections were incubated overnight at 4°C with the respective primary monoclonal antibody, * anti-p50, -p65, and IκBα dilutions (1:150, 1:50, and 1:50, respectively). Immunohistochemical staining was performed using the streptavidin-biotin complex (strept-ABC) method. All sections were then counterstained with hematoxylin.

Stereologic investigation

Stereology is a method that utilizes random, systematic sampling to provide unbiased and quantitative data. It provides practical techniques for extracting quantitative information about a three-dimensional material from measurements conducted with two-dimensional planar sections of the material. In healthy and diseased gingival tissues, the numerical density of cells (according to the antibodies’ stains) was evaluated stereologically. Stereological analyses were performed in a stereology workstation consisting of a modified light microscope (Leica DM4000 B), a motorized specimen stage for automatic sampling (BioPrecision MAC 5000 controller system), a CCD color video camera (Optronics MicroFire), and stereology software (Stereo Investigator version 6.0, Microbrightfield, Colchester, VT). The unbiased counting frame-fractionator combination is a stereological method for counting cells in tissue sections.²⁵,²⁶ In our study, we used the unbiased counting frame and fractionator methods to estimate numerical density of cells according to staining with antibodies in the gingiva. Each glass microscope slide was sampled using the fractionator principle of the stereology software (Figure 1). Cells were counted using a 40x Leica Plan Apo objective (NA=1.40), which allowed accurate recognition (Figure 1). Each cell was counted by the stereology software according to the unbiased counting frame (Figure 1). The numerical density was estimated according to the formula given below:

\[ N_d = \frac{TM}{CFA \times NSS} \]

where Nd is numerical density, TM is total markers, CFA is counting frame area (XY) [μm²], and NSS is number of sampling sites.
Statistical analysis

A one-way analysis of variance (ANOVA) and a multiple range least significant difference (LSD) were used for intergroup comparisons (P=0.05). The coefficient of error (CE) for the numerical density estimation was the last calculated value. The generally accepted highest limit of CE is 5%. In our study, the coefficient of error for the numerical density was found to be 0.05.

RESULTS

The clinical periodontal scores of the patients are given in Table 1. The present study evaluated the nuclear and cytoplasmic NF-κB activations in both epithelial and connective tissue cells of the periodontitic and clinically healthy tissues. The differences between the stained cells of diseased and healthy gingival tissues are shown in Figure 1.
Table 2 shows the comparisons of the cytoplasmic and nuclear immunoreactivities of p50, p65, and IkB in healthy and diseased periodontal tissues. According to the immunohistochemical analysis, there was a statistically significant increase in the numerical density values of the cytoplasmic positive stained cells of IkB in the case group, compared to the control group (P < 0.001). Statistically significant differences were found between the diseased and control groups when comparing the cytoplasmic and nuclear positive immunoreactivities of p50 (P < 0.05). Similarly, a statistically significant increase was found in the numerical density values of the nuclear positive stained cells of p65 in the diseased group compared to controls (P < 0.05). Statistically significant correlations were also found between the clinical periodontal scores and the immunohistochemical results of the diseased subjects (P < 0.001; Table 3).

### DISCUSSION

Chronic periodontitis is one of the most prevalent forms of periodontal diseases. An infectious disease, chronic periodontitis results in an inflammation within the supporting tissues of the teeth, progressive attachment loss, and bone loss. As with other chronic inflammatory diseases, chronic periodontitis is also related to several inflammatory molecules that act in the immune and inflammatory process. However, little is known about the molecular-level changes that are associated with the host response in chronic periodontitis. Both the host and the bacteria in the periodontal biofilm release proteolytic enzymes that cause tissue damage. Several products associated with NF-κB activation (especially IL-1, TNFα, and RANKL) are found in abundance in the diseased periodontium. Studies have concluded that RANKL proteins have a constant trigger circle with other NF-κB stimulators such as TNF-α and IL-1β and IL-6. Additionally, studies have found that RANKL-mediated osteoclastogenesis plays a pivotal role in inflammatory bone resorption.23 Kawai et al.23 showed the enhanced concentration of RANKL and IL-1β in periodontally diseased tissues. TNFα and IL-1β

### Table 1. The mean clinical periodontal scores of the subjects in case and control groups.

|    | Control mm±SD | Case mm±SD | P value |
|----|---------------|------------|---------|
| PI | 0.31±0.15     | 2.73±0.38  | *       |
| GI | 0.26±0.08     | 2.12±0.37  | *       |
| PD | 1.23±0.26     | 4.21±1.09  | *       |
| CAL| 0.00±0.00     | 4.38±1.03  | *       |

*: Statistically significant differences within the groups (P < 0.001).

### Table 2. Comparison of cytoplasmic and nuclear immunoreactivity of p50, p65 and IkB in healthy and diseased periodontal tissues.

|        | Healthy tissues | Diseased tissues | P value |
|--------|-----------------|-----------------|---------|
| IkB    |                 |                 |         |
| Cytoplasmic immunoreactivity | 1.2x10^-4 ± 0.7x10^-4 | 4.4x10^-4 ± 2.5x10^-4 | *       |
| p50    |                 |                 |         |
| Cytoplasmic immunoreactivity | 1.4x10^-4 ± 1.0x10^-4 | 3.6x10^-4 ± 2.9x10^-4 | *       |
| Nuclear immunoreactivity     | 0.3x10^-4 ± 0.2x10^-4 | 1.0x10^-4 ± 0.8x10^-4 | *       |
| p65    |                 |                 |         |
| Cytoplasmic immunoreactivity | 1.2x10^-4 ± 0.9x10^-4 | 6.4x10^-4 ± 3.2x10^-4 | *       |
| Nuclear immunoreactivity     | 0.3x10^-4 ± 0.2x10^-4 | 2.7x10^-4 ± 1.7x10^-4 | *       |

*: Statistically significant differences within the groups (P < 0.05).

MND: Mean numerical density of the stained cells
can stimulate osteoclast differentiation in a synergistic fashion and are known to control their own expression. An increase of these proinflammatory cytokines in the tissues causes the re-production of RANKL, TNFα, and IL-1β from the osteoblasts. RANKL proteins released from osteoblasts induce their own receptors on the osteoclasts and the receptor activator NF-κB (RANK), and they regulate RANKL-mediated osteoclastogenesis. On the other hand, TNFα and IL-1β activate NF-κB in the immature osteoclasts and stimulate the formation of a mature osteoclast. Hence, understanding of the role of NF-κB activation in chronic inflammation may form a basis for future strategies in the treatment of periodontal diseases.

This study evaluated and compared the expression transcription factor NF-κB in the gingival tissues of both healthy and chronic periodontitis patients. According to stereologic examinations, a striking increment was found in both the cytoplasmic immunoreactivity of IkBα [P<.001] and the nuclear and cytoplasmic expression NF-κB [p50-p65] [P<.05] in diseased tissues, compared to controls. Over-expression of NF-κB in periodontally diseased tissues explains the molecular signaling mechanism in the initiation and progression of the disease. Moreover, statistically significant correlations were found between the periodontal scores (including probing depth and clinical attachment level) and the NF-κB activation in diseased subjects [P<.001]. The compatibility between the level of clinical periodontal scores and NF-κB and IkBα expression provides evidence of the effectiveness of NF-κB activation in chronic periodontal inflammation. However, several systemic conditions linked with periodontitis, including atherosclerosis and diabetes mellitus, have also been reported to enhance NF-κB activation. In vitro studies have demonstrated NF-κB activation in periodontal tissues and by periodontopathogens, including Porphyromonas gingivalis. Moreover, smoking (a risk factor for periodontitis) has recently been recognized as an activator of NF-κB. NF-κB is an essential factor in controlling both immune and inflammatory responses. Remarkably, a large number of genes appear to be targets for activation by NF-κB, such as adhesion molecules [e.g., intercellular adhesion molecule-1 (ICAM-1) and vascular-cell adhesion molecule-1 (VCAM-1)], proinflammatory cytokines [e.g., TNFα, IL-1 and IL-6], proteinases (matrix metalloproteinase-1 (MMP-1) and MMP-9), and inflammatory enzymes [e.g., inducible nitric oxide synthase (iNOS)], which play pivotal roles in the chronic inflammatory process.

Chronic inflammation perpetuates and amplifies itself through the numerous autocrine and paracrine loops of cytokines, acting on the cells within a lesion. The cells within the inflammatory lesion are subjected to many extracellular stimuli, whereas only a handful of inducible transcription factors (including NF-κB) appear to play crucial roles in the regulation of inflammatory genes. Recently, studies have been directed toward producing specific NF-κB inhibitors, such as NBD peptide and IkBα superrepressor, in order to alter the host response in the immune and inflammatory processes. The pathway can be broken by neutralizing the biological activities of extracellular inflammatory mediators or by inhibiting cytokine production. Because NF-κB is the most important inducer of these circles, inhibition of NF-κB may provide an efficacious therapeutic strategy. In the present study, statistically significant activations of p50 and p65 were found in chronic periodontitis groups, compared to controls. However, the cytoplasmic activity of NF-κB was observed to be higher in the diseased group. A correlation between the clinical scores and the NF-κB activation was also significant when considering the role of NF-κB in the disease progression.

Table 3. Correlations between the clinical periodontal scores and immunohistochemical results of the diseased subjects.

|         | PI     | GI     | PD    | CAL    |
|---------|--------|--------|-------|--------|
| IkBα-cytoplasmic | ns     | ns     | 0.807** | 0.901** |
| p50-cytoplasmic   | ns     | ns     | 0.703** | 0.844** |
| p50-nuclear       | ns     | ns     | 0.517*  | 0.503*  |
| p65-cytoplasmic   | ns     | ns     | 0.898** | 0.721** |
| p65-nuclear       | ns     | ns     | 0.554*  | 0.541*  |

*: P<.05; **: P<.001; ns: not significant
**CONCLUSIONS**

It was shown that NF-κB was highly activated in subjects who had chronic periodontitis, compared to healthy controls. The findings of this study can be useful in planning new treatment strategies for periodontal diseases. Further investigations are needed to understand more about the signaling mechanisms of inflammatory mediators and their interactions with NF-κB in chronic periodontitis.

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