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Toll-like Receptor 4 Polymorphism and Periodontitis in Korean Population

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The primary cause of periodontitis is plaque-associated anaerobic gram-negative bacteria. As shown in the patients with defects in the number or function of neutrophils, innate immunity plays an important role in resistance to bacterial infection and periodontitis. Toll-like receptor 4 (TLR4) is one of the key receptors that recognize the molecular patterns of microbes and initiate innate immune response. To understand the role of TLR4 in the pathogenesis of periodontitis, we investigated whether Asp299Gly of TLR4 mutation is associated with periodontitis in Korean population. Subjects for this study included 90 healthy subjects and 98 periodontitis patients. The Asp299Gly mutation was screened by PCR-Restriction Fragment Length Polymorphism (RFLP) of genomic DNA from blood cells using a primer that creates a *Nco*I restriction site only in the mutant allele. The Asp299Gly mutation was not found in all subjects tested. Our results suggest that the Asp299Gly mutation of TLR4 is very rare in a Korean population. Further mutation screening may be required to determine the role of TLR4 in the pathogenesis of periodontitis.

Keywords: Toll-like receptor 4, Periodontitis, Innate immunity, Polymorphism.

Introduction

Periodontitis is an inflammatory disease caused by plaque-associated anaerobic gram-negative bacteria. As shown in the patients with defects in the number or function of neutrophils, innate immunity plays an important role in resistance to bacterial infection and periodontitis (Kornman *et al*, 1997). Innate immune mechanisms act immediately because they are ready to recognize and eliminate microbes, thus provide the front line of defense for infections. The instant action of innate immunity depends on the several characteristics of the receptors for microbes. First, all cells of a particular type, i.e. neutrophils express identical receptors, and cells do not need clonal expansion. Second, the receptors, called pattern-recognition receptors (PRRs), have limited diversity but detect most or all microbes because they recognize pathogen-associated molecular patterns (PAMPs) shared by classes of microbes, such as LPS, unmethylated CpG DNA, lipoteichoic acid, peptidoglycan, and double stranded viral RNA (Zhang *et al*, 2004). A set of Toll-like receptors (TLRs), human homologs of *Drosophila* Toll protein, has been identified as PRRs. TLRs are transmembrane receptors with an extracellular leucine-rich repeat domain and an intracellular signaling domain (Smirnova *et al*, 2000). The extracellular domain binds to various ligands directly or indirectly through interaction with other proteins such as LPS-binding protein, CD14, and MD2 (Kritzik *et al*, 2001; Armant *et al*, 2002). Signaling through TLRs results in the activation of transcription factors including nuclear factor-κB (NF-κB), which induces inflammatory responses, non-specific pathogen resistance, and the up-regulation of co-stimulatory molecules, ultimately leading to the activation of adaptive immune response (Hallman *et al*, 2001).

Among 11 of TLR members, TLR4 has the most important function in defense to gram-negative bacteria. The ligands for TLR4 include LPS from gram-negative bacteria,

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lipoteichoic acids from Gram-positive bacteria, taxol from plant, and F protein from respiratory syncytial virus (Shizuo *et al*, 2003). First evidence for association between LPS signaling and TLR4 was provided by the studies of C3H/HeJ and C57BL/10ScCr mice with known hypo-responsiveness to LPS. The two different inbred strains had distinct mutations involving the same gene, TLR4 (Poltorak *et al*, 1998). Defective LPS signaling in mice rendered them resistance to septic shock yet highly susceptible to gram-negative infection (Poltorak *et al*, 1998). Arbour *et al*, (2000) identified common mutations (Asp299Gly and Tlr399Ile) in a human TLR4 gene and showed that Asp299Gly mutation was associated with hypo-responsiveness to LPS. More recently, those mutations were reported to be associated with an increased risk for gram-negative bacterial infections (Agnese *et al*, 2002).

Although periodontitis is initiated by plaque-associated bacteria, the progression of disease is influenced by many other factors such as the virulence of infected bacteria, age, smoking, economic status, race, and genetic factors. Since TLR4 has a central role in mediating innate immune response to periodontopathic bacteria, we investigated whether the Asp299Gly mutation of TLR4 gene is associated with periodontitis in Korean population.

Materials and Methods

Subjects

Subjects for this study included 90 healthy control subjects, 23 patients with aggressive periodontitis, 34 with moderate and 41 with severe chronic periodontitis, all of whom were Koreans who have visited the Department of Periodontology at Seoul National University Dental Hospital (Table 1). The study was approved by the Institute Review Board at Seoul National University and a written informed consent was obtained from all subjects.

Clinical evaluation

Clinical parameters including probing depth (PD), clinical attachment loss (CAL), bleeding on probing (BOP), gingival index (GI), supragingival plaque accumulation (PI) were assessed. Control subjects showed neither CAL nor PD greater than 4 mm at more than one sites. The severity of chronic periodontitis is based on the amount of CAL and is designated as moderate (4-6 mm CAL) or severe (>6 mm CAL). Clinical criteria for aggressive periodontitis were defined as follows: $\geq$5 mm attachment loss at more than one site on more than $\geq$8 teeth, at least three of which were not first molars and incisors, and disease onset before the age of 35 years.

Probing depth and clinical attachment loss were recorded using the Florida Probe® (Florida Probe Co., Gainesville, FL, USA) in 6 sites of each tooth. Full mouth radiographs were taken by digital X-ray to evaluate alveolar bone levels using V-works version 3.5 (Cybermed, Seoul, Korea).

Genomic DNA isolation

Genomic DNA was prepared from peripheral blood using the Puregene blood kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

TLR4 mutation screening

PCR was carried out in a total volume of 30 μl containing 1X PCR buffer, 1.25 U of *Taq* polymerase (TaKaRa Shuzo, Shiga, Japan), dNTPs mixture (0.2 μM), 0.3 μM of each primer, and 100 ng of DNA. The sequences of primers used are shown in Table 2. One nucleotide in the forward primer was altered to generate a restriction site for *Nco*I only in a mutant allele (Fig. 1). The amplifications were performed in a thermal cycler (GeneAmp PCR system 9600; Perkin-Elmer, Wellesley, MA, USA) using the following temperature profile: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min for 35 cycles with an initial denaturation at 94°C for 5 min and a final extension at 72°C for 5 min. For control of *Nco*I enzyme condition, we carried out PCR about exon 7 of T-box transcription factor gene TBX22 contained *Nco*I enzyme site (TBEX7 F 5'-CTG GGG ATG CTG AAA GTT GAC T-3'). The amplifications were performed in a thermal cycler using above temperature profile.

Five microliters of the PCR products were digested with

Table 2. Primer sequences for PCR and sequencing of TLR4

Forward	5'-AGC ATA CTT AGA CTA CCT CCA TG-3'
Reverse	5'-TTT ACC CTT TCA AGA GTC ACA CTC A-3'

C: altered nucleotide to create *Nco*I site in mutated gene

Table 1. Study population characteristics

Diagnosis	Gender		mean Age (yr)	mean Probing Pocket Depth (mm)	mean Clinical Attachment Loss (mm)	Supragingival Plaque Accumulation (%)	Bleeding on Probing (%)	mean Gingival Index
	Male	Female						
control	64	26	28	2.06	2.10	43.42	11.82	0.20
Aggressive	16	7	32	3.83	4.36	70.02	59.51	0.93
Chronic moderate	17	18	49	2.81	3.17	67.09	41.72	0.74
severe	24	16	52	3.69	4.37	69.32	55.22	0.91
Total	121+67=188							

*Nco*I (New England Biolabs, Hitchin, UK) at 37°C for two hours. Undigested and digested products were analyzed in parallel on 2.5% SYBR Green I dye (Roche, Mannheim, Germany) - stained agarose gels and visualized using a Las-1000 Plus luminescent image analyzer (Fuji, Tokyo, Japan).

Sequencing of PCR products

To confirm the sequence of PCR products, the PCR products were purified with the QIAquick PCR purification kit (Qiagen, Chatworth, CA, USA), and sequenced at TaKaRa Korea Biomedical Inc., Seoul, Korea.

Results

Table 1 shows the characteristics of 188 subjects included in this study. The genotyping was screened by PCR-RFLP using a primer that creates a *Nco*I restriction site only in a mutant allele (Fig. 1). As shown in Figure 2B, all PCR products following *Nco*I digestion produced a single band with the expected size of 102 bp, indicating no Asp299Gly mutation in all subjects studied. As control, the 192 bp fragment of TBX7 gene including a *Nco*I restriction site was amplified and digested with *Nco*I in same condition, producing 144 bp and 48bp fragments (Fig. 2A). And we confirmed that PCR products had the nucleotide altered to

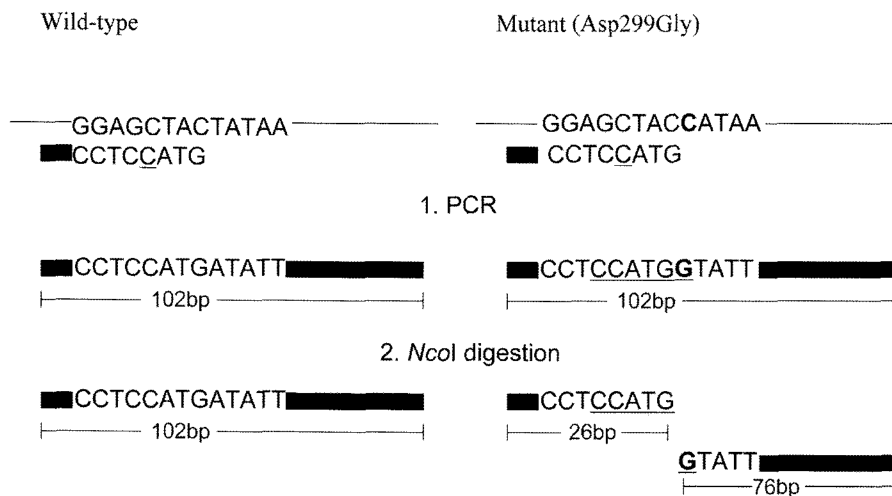

Figure 1. Schematic diagram of TLR4 mutation screening. First, PCR using a primer with a single nucleotide change (Table 2), second, *Nco*I digestion of PCR products. The PCR products of wild-type will give a single fragment with 102 bp. The PCR products of the Asp299Gly mutant will give two fragments with the 26 bp and 76 bp.

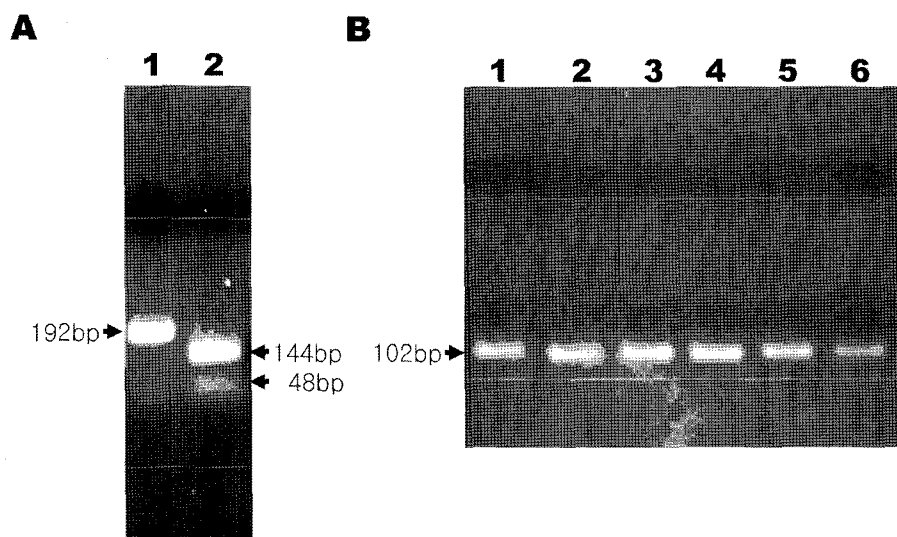

Figure 2. Screening of the Asp299Gly mutation in human TLR4. A. Control of *Nco*I enzyme condition. Lane 1: PCR product of TBX gene before *Nco*I digestion, Lane 2: PCR product of TBX gene after *Nco*I digestion, B. Lanes 1-6: PCR products of *Nco*I digest. All PCR products were shown as a single band after digestion with *Nco*I.

create a *Nco*I by sequencing analysis.

Discussion

Periodontitis is a multi-factorial infectious disease and the role of host immune response in the pathogenesis of the disease has long been discussed. The role of innate immunity in periodontitis can be considered in two opposite aspects: defense to causative bacteria and tissue destruction through inflammatory process. Innate immunity forms an epithelial barrier that defend the invasion of bacteria physically and biochemically through the secretion of antimicrobial peptides (Dixon *et al*, 2004). In addition, phagocytes in innate immune system, especially neutrophils that emigrate into periodontal pockets, are crucial for the clearance of bacteria (Madianos *et al*, 1997). On the other hand, recruitment of phagocytes and clearance of bacteria by those phagocytes are accompanied by the production of proinflammatory cytokines, inflammatory mediators, and various proteinases. The contribution of IL-1, TNF-α, and PGE_2 to osteoclast differentiation is well known (Bradstrom *et al*, 2001). In a *Macaca fascicularis* primate model of experimental periodontitis, soluble receptors to IL-1 and TNF-α inhibited the recruitment of inflammatory cells, osteoclastogenesis, and alveolar bone loss (Assuma *et al*, 1998). Nevertheless, people with defects in the number, migration, or function of phagocytes are susceptible to severe periodontitis, suggesting that the role of phagocytes in defense is greater than that in tissue destruction (Gronert *et al*, 2004).

The innate immune system detects the presence of bacteria by PRRs and TLR4 has a pivotal role in the recognition of gram-negative bacteria. TLR4 recognizes the LPS of gram-negative bacteria as PAMP through interaction with LBP/CD14/MD2. The role of TLR4 in the pathogenesis of periodontitis can be viewed in two aspects, too. TLR4 signaling induces the up-regulation of various anti-microbial peptides directly or indirectly through the production of pro-inflammatory cytokines, IL-1 and TNF-α (Hancock *et al*, 2000). Lack of LL37 in the neutrophils of patients with Kostmann syndrome led to early onset periodontitis, indicating the importance of anti-microbial peptides in resistance to periodontitis (Pitsep *et al*, 2002). However, IL-1 and TNF-α induced by TLR4 signaling can aggravate tissue destruction. There are several reports that the polymorphic allele of high IL-1β producer is associated with severe or early onset periodontitis although some groups did not find any association (Hodge *et al*, 2001; Tai *et al*, 2002). Therefore, how the dysfunction of TLR4 will affect the pathogenesis of periodontitis, is difficult to predict.

Since the common mutations (Asp299Gly, Thr399Ile) of TLR4 in human have been reported, many groups studied the association between the TLR4 polymorphism and diseases in which TLR4 mediated immune response may have a role. The TLR4 mutations were associated with an increased incidence of gram-negative infections in critically ill patients or with severe respiratory syncytial virus bronchiolitis, but not with likelihood or severity of meningococcal disease (Read *et al*, 2001; Tai *et al*, 2004; Doreen *et al*, 2002). TLR4 is a major receptor for inflammatory stimuli potentially involved in the pathogenesis of atherosclerosis. Asp299Gly polymorphism was associated with a decreased risk of atherosclerosis and that of acute coronary event (Kiechl *et al*, 2002; Ameziane *et al*, 2003). It is interesting that Asp299Gly mutation is associated with Crohn's disease and ulcerative colitis (Franchimont *et al*, 2004). Three mutations in NOD2, a cytosolic PRR, are associated with Crohn's disease, a common inflammatory disease of the intestinal tract (Inohara *et al*, 2003). Defective NF-κB activation in response to the muramyl dipeptide of intestinal bacteria may result in reduced production of defensin from intestinal crypts and the failure of controlling intestinal bacteria in proper level (Inohara *et al*, 2003). TLR4 mutations may have a similar effect on defensin production and contribute to the pathogenesis of periodontitis.

None of the 188 Korean subjects that we screened had Asp299Gly mutation. The allele frequency of Asp299Gly varies depending on ethnic group: 3.3% to 7% in Caucasian, 6.7% in African American, 4.2% in Cameroonian, but 0% in Hispanic American, Japanese, and Melanesian (Lorenze *et al*, 2001; Ferrand *et al*, 2002; Smirnova *et al*, 2001; Sanchez *et al*, 2004; Hise *et al*, 2003; Hise *et al*, 2003; Okayama *et al*, 2002). While we were doing screening, Folwaczny *et al*. (2004) reported that the allele frequencies of the Asp299Gly mutation in healthy subjects and patients with periodontitis were not significantly different, being 4.1% and 3.3%, respectively (Folwaczny *et al*, 2004). The beneficial and the deleterious results of TLR4 signaling might offset each other in the pathogenesis of periodontitis. When the entire coding region of TLR4 in 348 individuals from several population samples was sequenced, 12 amino acid variants including Asp299Gly and Thr399Ile were identified. Furthermore, the frequencies of the amino acid variants were significantly lower compared to those of mutations in intron or silent mutations. It is proposed that weak purifying selection acted on TLR4 and most mutation affecting TLR4 protein may have at least mildly deleterious phenotypic effects (Smirnova *et al*, 2001). Therefore, to exclude the role of TLR4 mutations in the pathogenesis of periodontitis completely, further studies involving other TLR4 variants are required.

In conclusion, the Asp299Gly mutation of TLR4 was not associated with periodontitis in Koreans. However, the presence of other TLR4 variants in Koreans and the role of other known TLR4 variants in periodontitis remain to be answered, yet.

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