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A Hyperactive Neutrophil Phenotype in Aggressive Periodontitis

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Although neutrophils function in both defense and tissue destruction, their defensive roles have rarely been studied in association with periodontitis. We hypothesized that peripheral neutrophils are pre-activated *in vivo* in periodontitis and that hyperactive neutrophils would show enhanced phagocytic ability as well as an increased production of reactive oxygen species (ROS). Peripheral blood neutrophils from patients with aggressive periodontitis and age/gender-matched healthy subjects (10 pairs) were isolated. The levels of CD11b and CD64 expression on the neutrophils and the level of plasma endotoxin were determined by flow cytometry and a limulus amebocyte lysate test, respectively. In addition, neutrophils were subjected to a flow cytometric phagocytosis assay and luminol-enhanced chemiluminescence for non-opsonized *Fusobacterium nucleatum* in parallel. The neutrophilsfrom most patients expressed increased levels of both CD11b and CD64. In addition, the plasma from these patients tended to contain a higher level of endotoxin than the healthy controls. In contrast, no differences were found between the two groups with regard to phagocytosis or ROS generation by *F. nucleatum*. The ability to phagocytose *F. nucleatum* was found to positively correlate with the ability to produce ROS. In conclusion, peripheral neutrophils from patients with aggressive periodontitis are hyperactive but not hyperreactive to *F. nucleatum*.

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Introduction

Periodontitis is an inflammatory disease that leads to the destruction of hard and soft periodontal tissues. Although periodontal disease is initiated by plaque-associated bacteria, it is widely accepted that tissue destruction is mostly mediated by host immune responses to bacteria rather than by bacteria themselves. Periodontal lesions are characterized by increased infiltration of various inflammatory cells and increased oxidative stress [1]. Neutrophil is one of the inflammatory cells that are thought to be involved in periodontal tissue destruction because activated neutrophil releases a variety of tissue-damaging molecules including elastase, matrix metalloproteinases, reactive oxygen species (ROS), and inflammatory cytokines [1].

Over the past few decades, many researchers have studied the ability of peripheral blood neutrophils to produce ROS using a variety of stimulations. At present, there is a general consensus that peripheral blood neutrophils in patients with aggressive or chronic periodontitis produce higher levels of ROS than do healthy subjects in response to FcγR stimulation [2-4]. This hyperreactive phenotype has been reported to be a constitutive property of neutrophils in periodontitis because the hyperreactivity decreased, but nevertheless remained, even after treatment of periodontitis [5,6].

Although robust evidence supports the hyperreactivity of neutrophils to FcγR stimulation in periodontitis, the underlying basis has yet to be clarified. Several groups have studied the expression of constitutively expressed FcγRII (CD32), FcγRIIIb (CD16), and phox genes but found no differences between patients and controls [7]. Contradictory results have

been reported regarding the polymorphisms of FcγRII and FcγRIII genes. Specifically, FcγRII a-R/R131 and FcγRIIIb-NA2 were associated with disease recurrence and severity in Japanese patients, while FcγRII a-H/H131 and FcγRIIIb-NA1 were associated with periodontitis in Caucasians [8-11]. As the basis for hyperreactivity, potential *in vivo* priming had been suggested because patient neutrophils expressed lower percentages of CD62L and responded less effectively to priming with LPS than did neutrophils from healthy subjects [3,12]. Activated neutrophils rapidly shed CD62L, mobilize secretory vesicles, degranulate, and activate transcriptional programs [13,14]. CD11b, a component of CR3 (also known as Mac-1), is upregulated by the mobilization of secretory vesicles, which are triggered by various stimuli that include LPS, fMLP, leukotriene B4, platelet activating factor, IL-8, GM-CSF, and TNFα [15]. Pro-inflammatory cytokines such as IFN-γ and G-CSF induce the expression of FcγR I (CD64), which is expressed at very low levels on resting neutrophils [16]. Recently, the high number of CD64 molecules on the surface of peripheral blood neutrophils has been proposed as a sensitive marker for bacterial infection [16]. The level of CD64 on neutrophils also showed increases in chronic inflammation, including periodontitis [17]. Therefore, the levels of CD64 and CD11b would be good markers to determine whether or not neutrophils are pre-activated *in vivo*.

Although neutrophils have roles in both defense and tissue destruction, the defensive aspect has rarely been studied in association with periodontitis. We hypothesized that peripheral neutrophils in periodontitis are pre-activated *in vivo* and that hyperactive neutrophils would show enhanced phagocytic ability as well as increased production of reactive oxygen species (ROS). Most previous studies stimulated neutrophils using serum- or IgG-opsonized *Staphylococcus aureus* that is irrelevant to periodontitis. Matthews *et al*. reported that peripheral neutrophils from periodontitis patients release excess ROS following stimulation with non-opsonized *Fusobacterium nucleatum* [4]. *F. nucleatum* plays an important role in the colonization of periodontal pathogens [18], is highly susceptible to phagocytosis by neutrophils even in the absence of opsonization, and is a potent inducer of ROS production [19]. To test our hypothesis, we determined the levels of CD11b and CD64 on neutrophils as well as the level of endotoxin in plasma. In addition, we examined the abilities of peripheral blood neutrophils to phagocytose *F. nucleatum* and to produce ROS in response to *F. nucleatum* in parallel.

Materials and Methods

Subjects

Patients with aggressive periodontitis (n = 10, age range = 22-42 years) were recruited from patients who visited the department of periodontology at Seoul National University Dental Hospital. Age- and gender-matched periodontally healthy control subjects (n = 10, age range = 24-42 years) were recruited through an advertisement at Seoul National University Dental Hospital. Control subjects were designated as healthy if they had full-mouth probing pocket depth (PD) less than 3 mm. Exclusion criteria included a history of systemic disease, smoking, and medication of steroids or antibiotics within seven days. The study was performed according to ethical principles, after receiving approval from the Institutional Review Board of Seoul National University Dental Hospital. Written consents were obtained from all the subjects. After collecting peripheral blood in a heparin tube, PD, clinical attachment level (CAL), and bleeding on probing (BOP) were recorded by one calibrated examiner. All clinical parameters were measured with a manual probe (CP-12, Hu Friedy, Chicago, IL, USA). Each pair of a patient and a control subject was sampled on the same day and the paired samples were processed in parallel.

Isolation of plasma and neutrophils from human peripheral blood

Plasma sample was obtained from centrifugation of 1 ml blood and stored in - 20°C. The remaining 19 ml blood was layered on Ficoll-Hypaque (Amersham Bioscience, Uppsala, Sweden) after diluting in saline solution (0.9% NaCl) at 1:1 and centrifuged at 400 g for 40 min. Cell pellet containing neutrophils and RBC was subjected to hypotonic lyses of RBC three times as previously described [19]. The purity of neutrophils in recovered cells was greater than 95% as confirmed by staining cells with anti-CD15 mAb-FITC (BD Bioscience, San Diego, CA, USA).

Antibody stain and flow cytometry

Neutrophils (2×10^5 cells) were stained with anti-CD15-FITC (clone HI98), anti-CD11b-PE-Cy5 (clone ICRF44), and anti-CD64-PE (clone 10.1) antibodies, and analyzed by flow cytometry using a FACSCalibur (BD Biosciences). Neutrophils were gated based on forward cell scatter and side cell scatter. The expression level was calculated as the geometric mean fluorescence intensity (MFI) of the gated cells stained with specific antibodies subtracted by MFI of cells stained with isotype control antibodies.

Measurement of endotoxin in plasma

Plasma was diluted in entotoxin-free water at 1:10 and subjected to limulus amebocyte lysate (LAL) test using a kit (HyCult Biotech, Uden, Netherlands).

Bacteria preparation

F. nucleatum (ATCC 25586) was cultured in brain heart infusion broth (Difco) supplemented with 5 μg/ml hemin (Sigma, St. Louis, MO, USA) plus 10 μg/ml vitamin K (Sigma) at 37°C under an anaerobic atmosphere (5% H_2, 10% CO_2 and 85% N_2). Bacterial cells harvested in log phase were washed with PBS, fixed with 4% paraformaldehyde

(Sigma) for 20 min at room temperature, and then washed with PBS again. Half of the bacteria were stained with 5 μM 5 (and 6-)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probe, Eugene, OR, USA) in PBS at room temperature for 20 min. After washing twice with PBS, the concentration was determined by flow cytometry. The CFSE-labeled bacteria and unlabeled bacteria prepared in parallel were stocked in - 20°C. Bacteria prepared in one batch were used for all assays.

Phagocytosis assay

Human neutrophils (1×10^5 cells/500 μl/tube in triplicate) resuspended in RPMI medium supplemented with 10% heat inactivated FBS and 2 mM L-glutamine were mixed with labeled *F. nucleatum* at 1:25 and incubated at 37°C for 1 h. As a negative control, neutrophils fixed with 3.7% formaldehyde (Sigma) were incubated with the same amount of labeled bacteria. After incubation, the mixture was centrifuged at 4°C. Then, 500 μl trypan blue (400 mg/ml prepared in 0.85% saline solution) was added to quench the fluorescence of the bacteria bound on the surface and analyzed by flow cytometry. Efficient quenching was confirmed on negative control cells. Total 10000 events were acquired and data were analyzed using CellQuest software (BD Biosciences). Phagocytosis was expressed as the MFI of gated granulocytes.

Enhanced chemiluminescent assay for ROS

In 96-well plates, neutrophils (1×10^5 cells/100 μl), unlabeled *F. nucleatum* (2.5×10^6/50 μl), and luminol (50 μl of 2 mM solution, Sigma) were added. All cells and reagents were resuspended in RPMI medium supplemented with 10% heat inactivated FBS and 2 mM L-glutamine. As a negative and a positive control, neutrophils were stimulated with medium alone and 0.1 μM Phorbol 12-myristate 13-acetate (PMA, Sigma), respectively. After plating, the plate was immediately placed in a microplate luminometer (BMG Labtech, Ortenberg, Germany) and light emission in relative light units was recorded every 10 minutes during 110 minute incubation at 37°C. All samples were run in triplicate. ROS production was calculated by averaging the eleven measurements in triplicate.

Statistics

The Wilcoxon signed-rank test was used for all statistical comparisons of patients and controls. Spearman's rank correlation was used to determine association between two parameters. In this study, we could recruit only 10 pairs of patient and control subjects. To avoid a type II error that can occur due to small sample size, $P < 0.1$ was considered to be significant. All statistical analyses were carried out using SPSS Statistics 19 software (IBM Korea, Seoul, Korea).

Results

The expression levels of CD11b and CD64 on peripheral blood neutrophils from patients presented higher median values than did those from controls ($P = 0.07$ and $P = 0.06$, respectively, Fig. 1a and b). The levels of CD11b and CD64 had a strong positive correlation to each other ($P = 0.006$, Fig. 1c). The bivariate scatter plot of CD11b and CD64 revealed that neutrophils from most patients expressed increased levels of both CD11b and CD64.

In vivo upregulation of CD64 on neutrophils by intravenous injection of 2 ng/kg of *Escherichia coli* LPS was previously shown in humans [20]. Periodontitis patients experience bacteremia more frequently than do healthy individuals, contaminating blood with bacterial components. Thus, the levels of endotoxin in plasma were measured. The median value of endotoxin in plasma from patients was slightly higher than that from healthy subjects ($P = 0.07$, Fig. 2).

The ability of peripheral neutrophils to phagocytose *F. nucleatum* presented no difference between the control and patient groups ($P = 0.17$, Fig. 3). Similarly, the levels of ROS produced by stimulation with *F. nucleatum* were not different between two groups ($P = 0.36$, Fig. 4a). However, the levels of ROS produced by resting neutrophils or by

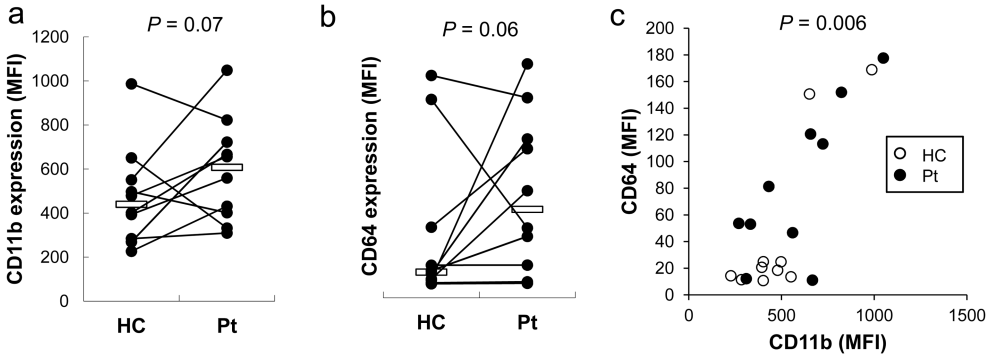

Fig. 1. Expression of CD11b and CD64 on peripheral neutrophils. Neutrophils isolated from peripheral blood were stained with (a) anti-CD11b-PE-Cy5 and (b) anti-CD64-PE monoclonal antibodies and analyzed by flow cytometry. Horizontal bars indicate median values. (c) Correlation between the levels of CD11b and CD64. Each patient (Pt) and age/gender matched healthy control (HC) subject has been connected with line.

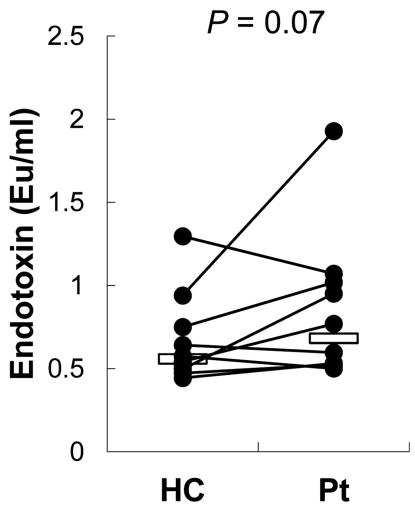

Fig. 2. The level of endotoxin in plasma. The level of endotoxin in plasma was measured by LAL test. Horizontal bars indicate median values. Each patient (Pt) and age/gender matched healthy control (HC) subject has been connected with line.

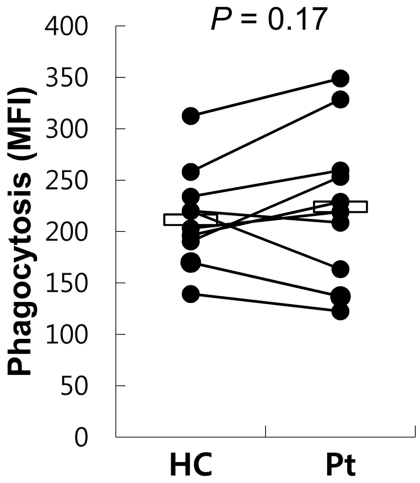

Fig. 3. Phagocytosis of *F. nucleatum* by neutrophils. Neutrophils were incubated with CFSE-labeled *F. nucleatum*. Phagocytosis was analyzed by flow cytometry after quenching the fluorescence of bacteria on the surface with trypan blue and expressed as the MFI of neutrophils. Horizontal bars indicate median values. Each patient (Pt) and age/gender matched healthy control (HC) subject has been connected with line.

stimulation with PMA were slightly higher in the patient group than those in the control group ($P = 0.06$ and $P = 0.07$, respectively, Fig. 4b, c). The ability to phagocytose *F. nucleatum* was positively correlated with the ability to produce ROS by stimulation with *F. nucleatum* ($P = 0.031$,

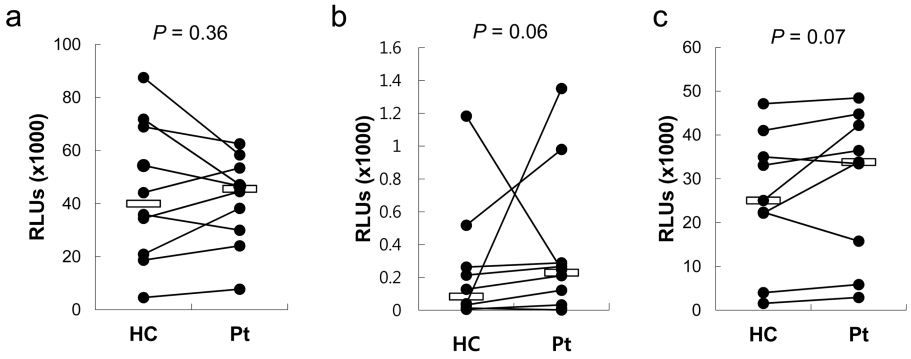

Fig. 4. Production of ROS by neutrophils. Total ROS production in the absence of stimulant (b) or by stimulation with *F. nucleatum* (a) or PMA (c) was measured by luminol-dependent chemiluminescence. Horizontal bars indicate median values. Each patient (Pt) and age/gender matched healthy control (HC) subject has been connected with line.

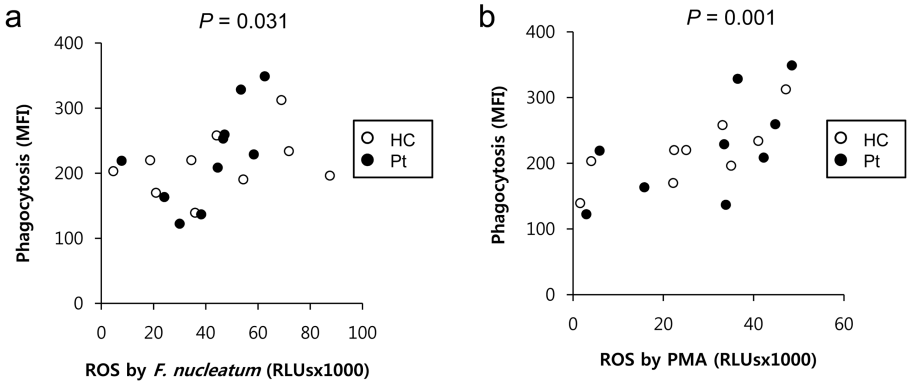

Fig. 5. Correlation between phagocytosis and ROS production. The ability of neutrophils to phagocytose *F. nucleatum* is plotted against the ability to produce ROS by stimulation with (a) *F. nucleatum* or (b) PMA.

Fig. 5a) or by PMA ($P = 0.001$, Fig. 5b).

Discussion

In this study, we addressed both the defensive and potentially detrimental aspects of neutrophil response to *F. nucleatum*, a key plaque-forming bacterium. Although, different from our hypothesis, peripheral neutrophils from patients with aggressive periodontitis and healthy subjects presented comparable abilities to phagocytose *F. nucleatum* and to produce ROS concomitantly, there was a significant positive correlation between phagocytosis and ROS production. The study by Matthews *et al*. also failed to show a significant increase in the level of total ROS produced by neutrophils from patients with chronic periodontitis in response to *F. nucleatum*, although there was increase in the level of extracellular ROS production by neutrophils from patients [4]. According to the literature, non-opsonized *S. aureus* induces no ROS from neutrophils [1]. Thus, IgG-coated *S. aureus* would be an optimal reagent to show the FcγR-dependent hyperreactivity of neutrophils. In contrast, non-opsonized *F. nucleatum* induces a substantial amount of ROS, although what kinds of receptors are involved in the *F. nucleatum*-induced ROS production is poorly understood. The presence of cations in assay buffer could have contributed to the result that showed no difference in ROS production between two groups. Cations in the buffer reduce the difference in ROS production between patients and controls [5] and most studies on ROS production have used Ca^{2+}- and Mg^{2+}-free PBS. However, we used RPMI supplemented with 10% FBS because we noticed that cations have an important role in the binding of non-opsonized bacteria to neutrophils [21]. Furthermore, reactions in RPMI buffer supplemented with 10% FBS would better reflect those in tissue fluids. These data indicate that peripheral neutrophils from patients with aggressive periodontitis are not hyperreactive to non-opsonized *F. nucleatum* in a cation-containing buffer.

Although we failed to observe the *F. nucleatum*-dependent hyperreactivity of peripheral neutrophils in aggressive periodontitis, our results indicate that neutrophils from patients are hyperactive. Similar to the results of other studies, neutrophils from patients produced slightly increased levels of ROS at basal state or by stimulation with PMA [7,22]. In addition, neutrophils from patients expressed increased levels of CD11b and CD64 on the surface. Low but significantly enhanced expression of CD64 in localized juvenile periodontitis was previously reported [7]. The level of CD11b was also higher in samples with localized juvenile and adult periodontitis than that in the control, although statistical significance was not achieved [3,7]. Thus, our results are in good agreement with those of earlier studies. Furthermore, a bivariate scatter plot of CD11b and CD64 revealed a clustering of most healthy subjects toward the lower left corner. Therefore, a combination of CD11b and CD64 may be a useful marker of neutrophils to segregate patients with periodontitis from healthy subjects. For that purpose, it is important to rule out any infection or chronic inflammation other than periodontitis. Viral infections also cause increased levels of CD64 on neutrophils [16]. Although we excluded subjects taking antibiotic medications, there is a possibility that our study population included some subjects with the seasonal flu or common cold.

The enhanced expression of CD11b and CD64 on neutrophils in periodontitis could be attributed to the contamination of blood with oral bacteria or bacterial components. Gentle mastication induced endotoxemia even in healthy individuals, and the induced endotoxin levels were significantly higher in patients with severe periodontitis than in healthy subjects [23]. We examined endotoxemia without mastication, and the levels of endotoxin were slightly higher in patients than in healthy controls. An epidemiologic study identified low-level endotoxemia as a strong risk factor for atherosclerosis and cardiovascular disease, particularly among smokers [24]. Low-level endotoxemia might be a key element that links smoking, periodontitis, and cardiovascular disease. Recently, *Veillonella* and *Streptococcus* were identified in the majority of atherosclerotic plaque samples [25]. Therefore, not only endotoxin but also any bacterial components, including those from Gram positive bacteria, that induce either IFN-γ or G-CSF from blood cells would contribute to the activation of neutrophils *in vivo*. Taken together, our results suggest that peripheral neutrophils in aggressive periodontitis seem to be pre-activated by bacterial components such as endotoxin.

The increased level of CD64 on the surface of neutrophils may be responsible for the FcγR-dependent hyperreactivity described in literature. Elevation in cytosolic free calcium ($[Ca^{2+}]i$) through the mobilization of Ca^{2+} from intracellular stores, followed by store-operated calcium entry from extracellular sources, is a prerequisite for the induction of assembly of nicotinamide adenine dinucleotide phosphate-oxidase components [26]. Among the FcγR expressed on neutrophils, only FcγR IIa and CD64 (FcγR I) can deliver activation signals via an immunoreceptor tyrosine-based activation-motif, which results in Ca^{2+} mobilization and respiratory burst [27]. Importantly, FcγR I is a high-affinity FcR, whereas FcγR II is a low-affinity FcR [27]. Several studies have addressed the possibility of *in vivo* priming by stimulation of isolated neutrophils with various reagents before subjecting them to an enhanced chemiluminescence assay [4,6,28]. The experimental conditions used in those studies were not optimal in simulating *in vivo* priming because the cells were stimulated in PBS for 30 min in the absence of NK cells and monocytes, the major producers of IFNγ and G-CSF, respectively. Nevertheless, priming of neutrophils with *F. nucleatum* and *Porphyromonas gingivalis* reduced FcγR-mediated hyperreactivity upon diagnosis and abolished it after therapy, suggesting the contribution of

peripheral priming to the observed hyperreactivity [6].

An important question is whether the hyperreactivity of neutrophils would be beneficial for the clearance of bacteria. Our study showed a positive correlation between the ability of neutrophils to phagocytose non-opsonized *F. nucleatum* and the ability to produce ROS. We previously reported a strong correlation between phagocytosis and ROS production in a study using three oral bacterial species, including *F. nucleatum* [19]. Opsonization of oral bacteria with IgG increased not only ROS production but also phagocytic uptake. It has been suggested that ROS can amplify FcγR-mediated phagocytosis in neutrophils [29]. Therefore, it is speculated that the FcγR-dependent hyperreactivity of peripheral neutrophils in periodontitis may also contribute to the clearance of bacteria.

In conclusion, peripheral neutrophils from patients with aggressive periodontitis had pre-activated phenotypes with increased expression of CD11b/CD64 and enhanced endotoxemia. The ability to phagocytose *F. nucleatum* has a positive correlation with the ability to produce ROS in response to *F. nucleatum*.

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