Induction of NADPH oxidases and antioxidant proteins by Porphyromonas gingivalis in KB cells

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I. INTRODUCTION

Periodontal diseases are a group of infections that lead to inflammation of the gingiva, destruction of the periodontal tissues, and loss of alveolar bone with eventual exfoliation of teeth. A variety of bacterial species are associated with the onset and progression of periodontal disease. In particular, evidence implicating a pathogenic role of gram-negative anaerobes including the species Porphyromonas gingivalis has been accumulated[1,2]. P. gingivalis can adhere to and invade epithelial cell, and may contribute to immune evasion and tissue destruction. The bacteria are a component of subgingival plaque that interfaces with epithelial cells lining the gingival sulcus.

Reactive oxygen species (ROS) are involved in the regulation of cellular activities ranging from host defense and inflammation to intracellular signaling and transcription, and they can be also generated as by-products of cellular metabolism in non-phagocytic cells[3,4]. Superoxide generation by phagocytes plays a crucial role in the elimination of invading microorganism. The NADPH oxidase complex catalyzes the transfer of an electron to molecular oxygen to form superoxide anion ($O_2^{-\bullet}$), which is then spontaneously or enzymatically converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD)[5,6]. The enzyme NADPH oxidase is composed of at least five protein components, including two transmembrane flavocytochrome b components ($gp91^{phox}$ and $p22^{phox}$) and four cytosolic components ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac protein)[7]. The family, so called the NOX gene family, currently consists of seven human homologs: NOX1, $gp91^{phox}$ (renamed NOX2), NOX3, NOX4, NOX5, DUOX1, and DUOX28). NOX1 is ex-

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pressed predominantly in large intestinal epithelial cells[9] and conserves the structural domains common to the catalytic core of phagocyte NADPH oxidase (gp91^{phox}, renamed NOX2), including the binding sites for heme, flavin, and NADPH[8,10]. NOX1 is a superoxide-generating enzyme that is activated by two novel proteins, that is NOXO1 (NAD(P)H oxidase organizer 1, p41^{nox}) and NOXA1 (NAD(P)H oxidase activator 1, p51nox)[11]. Most recently, it was found that HEp-2 human epithelial cells produce ROS that initiated the response in P. gingivalis[12]. Helicobacter pylori lipopolysaccharide activated Rac1 and transcription of NADPH oxidase NOX1 and its organizer NOXO1 in guinea pig gastric mucosa cells[13].

In order to fine-tune ROS level, and to protect themselves from oxidative stress, there are glutathione and the classical enzymatic antioxidants including, catalase and glutathione peroxidases (GPx), superoxide dismutase (SOD), peroxiredoxins (Prxs)[14,15]. P. gingivalis is subject to oxidative stress, not only from occasional exposure to air but also during neutrophil infiltration and oxidative burst or gum bleeding in advanced periodontitis[16]. It can be hypothesized that antioxidants involved in ROS scavenging have different distribution and expression in oral epithelial cells infected with P. gingivalis. They might be upregulated in antioxidant related inflammatory periodontal diseases, and they might play an important role in protecting epithelial cells against oxidants.

This study was aimed to investigate NADPH oxidase components in the generation of ROS and expression of antioxidant proteins in oral epithelial cells infected with P. gingivalis.

II. MATERIALS & METHODS

1. P. gingivalis culture

P. gingivalis strain ATCC 33277 was used in this study. The bacteria were cultured and maintained on anaerobic chamber in an atmosphere containing 5% CO_2, 10% H_2, 85% N_2 in brain heart infusion broth (BHI, Difco Laboratories, MI, U.S.A.) supplemented with 5 mg/ml of hemin (Tokyo Kasei Kogyo. Japan) and 0.5 mg/ml of Vitamin K (Sigma) at 37℃. The bacteria were harvested, washed in sterile 1x PBS Solution (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 (WelGENE Inc, Korea), and centrifuged (6,000×g for 20 min). The number of bacteria in the suspension was determined by the optical density (OD) at 600 nm. The bacteria were then centrifuged and resuspended in antibiotic-free Minimum Essential Medium Eagle (MEM) (WelGENE Inc, Korea) containing Earl's balanced salt solution (Hyclone, Logan, U.S.A.) supplemented with 5% fetal bovine serum (Gibco BRL Div, USA).

2. Epithelial cell culture

KB cells derived from an epidermoid cancer of the oral cavity were used. The cells were routinely cultured in humidified incubator with 95% air and 5% CO_2. The cells were inoculated into 10×10 cm tissue culture plates at a concentration of 1×106 cells/ml. Cells were used at 80 to 90% confluence for all experiments and infected with bacteria at a multiplicity of infection (MOI) of 100. This ratio overlaps the range reported in vivo for the association of P. gingivalis with epithelial cells[17].

3. MTT cytotoxicity assay

Cytotoxicity was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylte-trazolium bromide) colorimetric assay described by Mosmann T(1983)[18]. This method is based on the capacity of mitochondrial enzymes of viable cells to reduce the yellow soluble salt MTT (50 mg/ml, Sigma) to a blue insoluble formazan precipitate, which is quantified after dis-solution in an organic solvent. The blue for-mazan production correlates directly with the count of viable cells. In order to determine the cytotoxicity of P. gingivalis, KB cells were treated with P. gingivalis at an MOI of 100 for various times. Cultures of the control group were left untreated. Ten microliter of the MTT labeling reagent was added to each well, and the plates were incubated for 4h. One hundred microliter of the dimethyl sulfoxide was added to each well, and the cells were incubated for 5 -10 min. The absorbance was then measured with a microtiter plate reader (ELx 800UV, Bio-Tek Instruments. INC) at a test wave-length of 570 nm with a reference wave length of 690 nm. The OD was calculated as the dif-ference between the absorbance at the reference wavelength and that at the test wavelength. Percent viability was calculated as OD of bac-teria treated sample / control OD ×100.

4. Infection of epithelial cells

Approximately 1x105 cells were washed three times with 1x PBS and then infected by the ad-dition of a resuspended overnight culture of 107 P. gingivalis (ATCC 33277) in 1.0 ml of anti-biotic-free medium at 37℃. After a time of aerobic incubation, the monolayers were washed three times with PBS to remove extracellular bacteria. Medium containing gentamicin (300 μg/ml) and metronidazole (200 μg/ml) was then added to each well, and the plates were in-cubated for an additional 60 min aerobically at 37℃. After incubation, the cells were collected for detection of peroxredoxins (Prx I VI), su-peroxide dismutase (SOD) 1, 2, and 3 by Western blotting and NADPH oxidase (NOX) family, Rac1, 2 and cytosolic components ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$) by RT-PCR.

5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were prepared from cells using TRIzol® reagents (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Briefly, cells were washed in 1x DEPC-PBS (pH 7.4). Cells were removed by pipetting in the presence of 1 ml of TRIzol® reagents. Phase separation was accomplished by addition of 1-bromo-3-chloropropane (BCP) and cen-trifugation (12,000×g, 15 min). RNA was pre-cipitated using isopropanol and was quantitated by spectrophotometer. Two μg of the total RNA was reverse transcribed and PCR was done by RT-PCR premix (Bioneer, Korea) and PCR pre-mix (Klen-Taq PCR premix, Anygen, Korea). The primer sequences used are presented in Table 1. Amplification was performed starting with an initial denaturation step for 5 min at 94℃, followed by 35 cycles of denaturation for 30 s at 94℃ annealing for 30 s at 60℃ and extension for 30 s at 72℃ with a final ex-tension step for 7 min at 72℃.

6. Western blot analysis

Cells were lysated in a lysis buffer containing 150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 1% Nonidet P40, leupeptin (0.5 mg/ml), pepstatin (0.7 mg/ml), aprotinin (2mg/ml) and phenylmethylsufonyl fluoride (PMSF) (44 μg/ml). Cell lysates were centrifuged at 12,000×g for 20 min at 4℃ and the supernatant (20 μg of protein) was transferred and measured for protein concentrations using Bradford assay (Bio-Rad Laboratories, Hercules, CA). Twenty μg of denatured protein was separated by SDS-PAGE using an 12% polyacrylamide gel and transferred onto a polyvinylidine difluoride membrane (PVDF, Bio-Rad Laboratories, Hercules, CA). After nonspecific binding site were blocked with 5% (w/v) skim milk TBST solution containing 0.1% (vol/vol) Tween 20, the membrane were incubated for overnight with specific antibodies against peroxiredoxins (Prxs) Ⅰ-Ⅵ (1:2,000. LabFrontier, Seoul, Korea),

Table 1. Primer sequences used in RT- PCR

Gene	Primer sequence	RT-PCR Product (bp)
NOX1(NM-007052) sense antisense	 TCCCTGGAACAGGAGATGGA AATGCTGCATGACCAACACTCTTG	101
NOX2 (gp91^{phox}) (NM-000397) sense antisense	 TTTGTTCTTAGAACTGCTCCCATTTC CATTACCGAAGTTGAAGAGTTCTAAACAT	101
NOX4(AF532984) sense antisense	 AGTCCTCCGTTGGTTTGCA TTGACTGAGGTACAGCTGGATGTT	101
p22^{phox}(NM-000101) sense antisense	 TTGCCGACAGGATGCAGAA GCCGATCCACACGGAGTACTT	101
Rac 1(NM-199829) sense antisense	 TGCAGGCCATCAAGTGTGTGGT GCTGAGACATTTACAACAGCAGGCAT	645
Rac 2(AF498964) sense antisense	 ACACCACCAACGCCTTTCC CCACAGCCCCAGGTTCACT	101
p40^{phox}(NM-000631) sense antisense	 TCTTCCCTCTCTCCTTCGTGAA GGTGCTTGATGGTGTCTTCGTAGT'	101
p47^{phox}(NM-000265) sense antisense	 GCTCGAGGGTGAAGCTGTTG TGCAGGTACATGGACGGAAA	101
p67^{phox}(NM-00433) sense antisense	 TTTGGAAAGCACTCGGAGAGA GCAGAAGGGTGCTAAATCTTACAAAC	101

Table 1 continued. Primer sequences used in RT- PCR

Gene	Primer sequence	RT-PCR Product (bp)
NOXO1(AF532984) sense antisense	 CGAATTCAGGCAGCTCAAGAA GGTGCATCGAGAAGCTTTGG	101
NOXA1(NM-006647) sense antisense	 CGAATTCAGGCAGCTCAAGAA AGCACCGACCTCTGTCTGT	87
Prx 4(NM-006406) sense antisense	 GGTGGACAAGTGTACC CAGAGCATGCTACCAC	283
Prx 5(NM-012094) sense antisense	 CAAGGTGGGAGATGCC CAGGAGCCGAACCTTG	283
GAPDH sense antisense	 TGCATCCTGCACCACCAACT CGCCTGCTTCACCACCTTC	354

GPx (1:2,000. LabFrontier, Seoul, Korea), SOD1, 2 (1:2,000. LabFrontier, Seoul, Korea), SOD3 (1:1,000. Stressgen, Canada), followed by horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Cell Signaling technology, Beverly, USA). Immunoreactive signals were detected by West Save (Chemiluminescent detection kit for HRP, LabFrontier, Korea). After bound antibodies were removed, the membrane was reblotted using an anti-β-actin antibody. Densitometric analysis of bands was performed by Scion Image software (SCION corp. Frederick, MD). Data were expressed as mean ± SD of independent triplicate experiments.

III. RESULTS

1. Cytotoxicity of P. gingivalis on KB cells

The MTT assay was performed to assess the effect of P. gingivalis on cell viability of oral KB epithelial cells and to determine the appropriate concentration to be used for the treatment of cells. P. gingivalis (MOI 100) showed suppressive effect on the viability of KB cells, compared to the non-treated cells (Figure 1). The reduction of cell viability was observed since the 5 min of P. gingivalis infection.

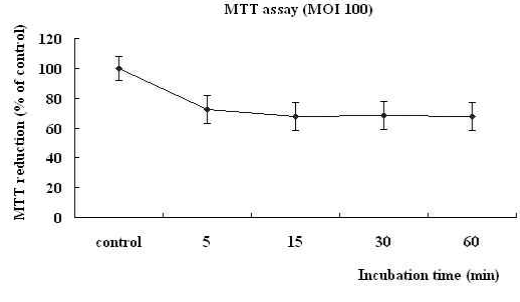

Figure 1. Cytotoxicity of P. gingivalis on KB cells. *P. gingivalis* strain ATCC 33277 was infected at an MOI of 100 and incubated for the time periods indicated. Cellular viability was determined by the MTT assay and expressed as percentage of the control value. The results are expressed as the mean ±SD from triplicate cultures.

2. Induction of NOX4 after P. gingivalis infection.

KB cells were infected with P. gingivalis for the different time period of 15, 30, and 60 min. The levels of P. gingivalis-stimulated membrane bounded NADPH oxidase (NOX1, NOX2 (gp91^{phox}), NOX4, and p22^{phox}) expression were measured by RT-PCR. As shown in Figure 2, NOX1 transcription decreased at 30min and recovered at 60 min. NOX2 (gp91^{phox}) transcription was not altered whereas NOX4 transcription increased. Transcription of p22^{phox} was not changed. Thus the O2- production induced by P. gingivalis infection appears to involve the enhanced expression of NOX4.

3. Expression of mRNA for cytosolic components of NADPH oxidase after P. gingivalis infection.

Expression of cytosolic components of NADPH oxidase (p40^{phox}, p47^{phox} and p67^{phox}) after P. gingivalis was examined by RT-PCR. The levels of p47^{phox} and p67^{phox} expression were not changed whereas p40^{phox} mRNA transcription decreased with P. gingivalis infection (Figure 3).

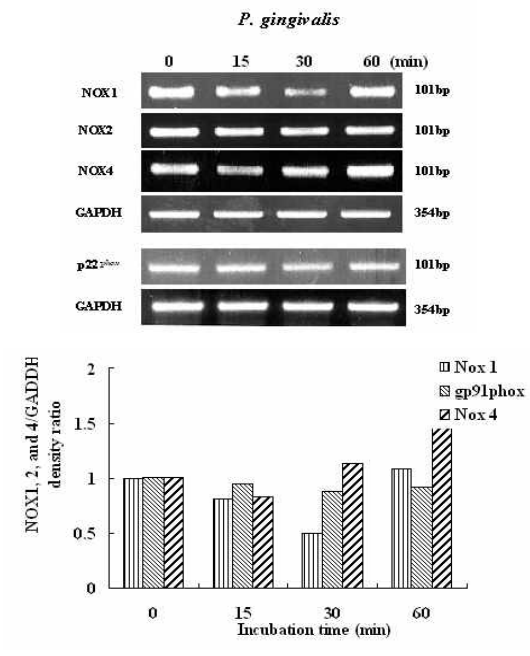

Figure 2. Expression of mRNA for NOX1, 2 and 4 in KB cells infected with P. gingivalis. KB cells were infected with P. gingivalis (MOI 100) and incubated for the indicated time periods, and total RNA was prepared. The expression levels of NOX1, NOX2, NOX4 and GAPDH mRNA were measured using RT-PCR.

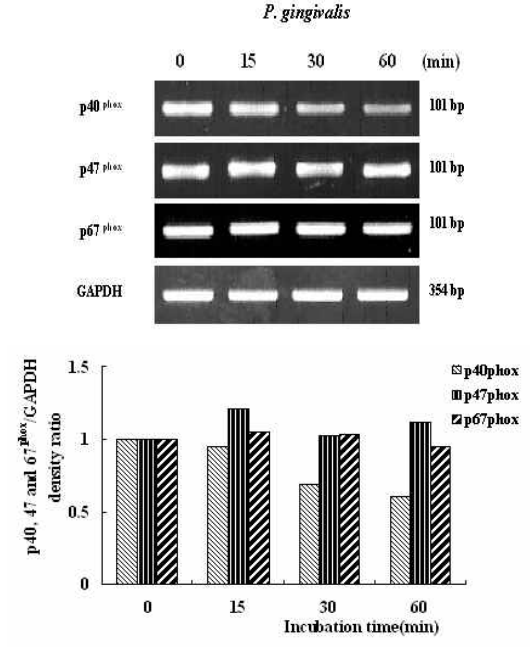

Figure 3. Expression of mRNA for cytosolic components of NADPH oxidase in KB cells infected with P. gingivalis treatment. KB cells were infected with P. gingivalis (MOI 100) and incubated for the indicated time periods, and total RNA was prepared. The expression level of mRNA for cytosolic components of NADPH oxidase was measured using RT-PCR.

4. Expression of NOXA1 and NOXO1 mRNA after *P. gingivalis infection.*

The expression of mRNA for NOX activating protein such as NOXA1 (NAD(P)H oxidase activator 1, p51nox $p67^{phox}$ homologue) and NOXO1 (NAD(P)H oxidase organizer 1, p41nox, $p47^{phox}$ homologue) was investigated in oral epithelial (KB) cells infected or uninfected with P. gingivalis. The expression of NOXA1 mRNA was decreased in a time-dependent manner but NOXO1 mRNA expression was increased (Figure 4). Thus P. gingivalis stimulated the expression of NOXO1 and NOX4 mRNA by KB cells

5. Induction of Rac1 and Rac2 mRNA after *P. gingivalis infection.*

Expression of mRNA for small GTPase-binding proteins (Rac1 and Rac2) was measured by RT-PCR. As shown in Figure 5, Rac1 transcripts were increased at 60 min of incubation. But Rac2 transcripts were not changed with P. gingivalis stimulation.

6. Expression of SODs after *P. gingivalis* infection.

The expression of SODs with P. gingivalis infection was investigated. As shown in Figure

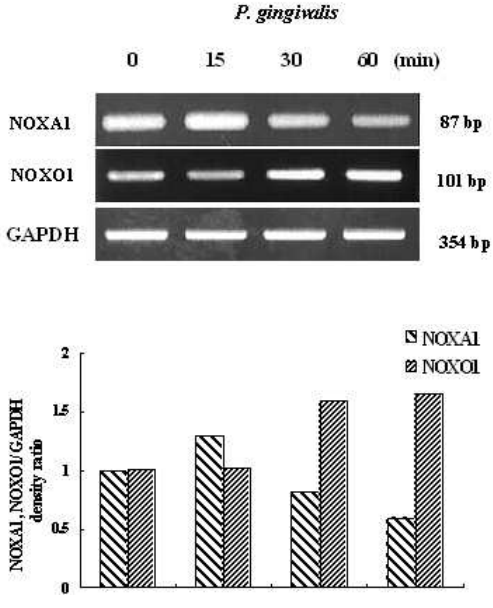

Figure 4. Expression of mRNA for NOXA1 and NOXO1 in KB cells infected with P. gingivalis. KB cells were infected with P. gingivalis (MOI 100) and incubated for the indicated time periods, and total RNA was prepared. The expression levels of NOXA1, NOXO1 and GAPDH mRNA were measured using RT-PCR.

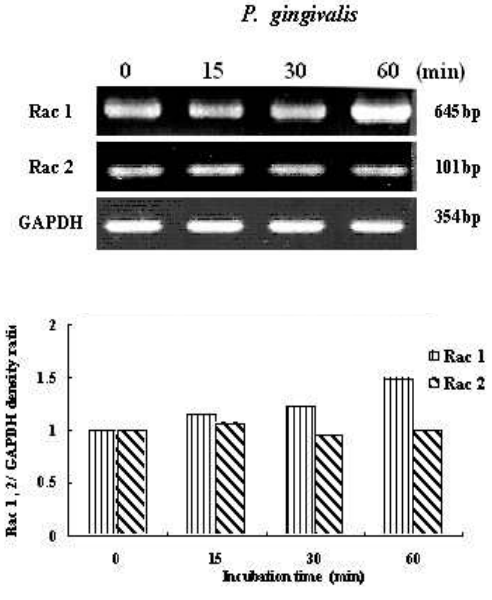

Figure 5. Expression of Rac1 and Rac2 mRNA in KB cells infected with P. gingivalis. P. gingivalis induced Rac1 mRNA expression in KB cells. KB cells were infected with P. gingivalis (MOI 100) and incubated for the indicated time periods, and total RNA was prepared. The expression levels of Rac1 and 2, and GAPDH mRNA were measured using RT-PCR.

6, when KB cells were infected with P. gingivalis, the expression levels of SOD1, 2, and 3 were increased in a time-dependent manner (two fold increase).

7. Expression of glutathione peroxidase (GPx) after P. gingivalis infection.

The expression of GPx with P. gingivalis was investigated in oral epithelial cell line (KB cells). As shown in Figure 7, when KB cells were infected with P. gingivalis, the expression of GPx was increased in a time-dependent manner. This GPx expression pattern was similar to that SOD.

8. Expressions of Prxs after P. gingivalis infection.

The expression of Prx antioxidant proteins was investigated by western blot analysis. Control KB cells expressed Prx I-VI. When KB cells were infected with P. gingivalis, the Prx II expression was 3 fold increased and the expression of other Prxs was not changed (Figure 8). However, the expression of PrxIV and PrxV was increased in a time-dependent manner in P. gingivalis infected KB cells. PrxIV expression was increased 4 times and PrxV expression was increased 6 times, compared to control (Figure 9).

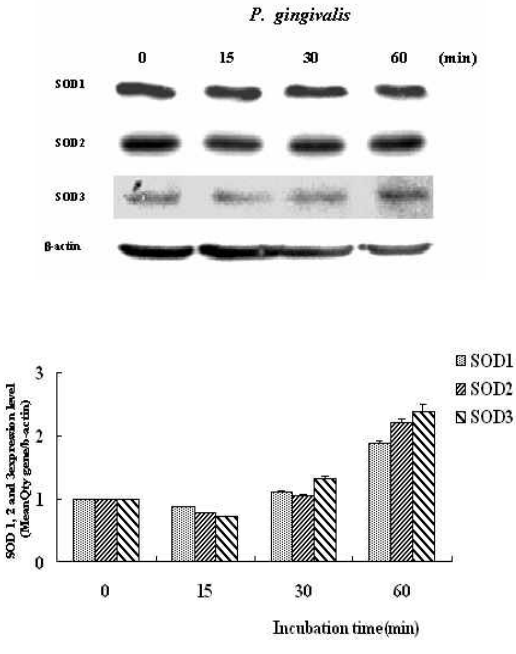

Figure 6. Expression of the SODs in KB cells infected with P. gingivalis. Western blot analysis of SOD1, 2 and 3 expression in KB cells infected with P. gingivalis at an MOI of 100 for the time periods indicated. Intensities were quantified by densitometry with the intensity of uninfected control cells set to 1.

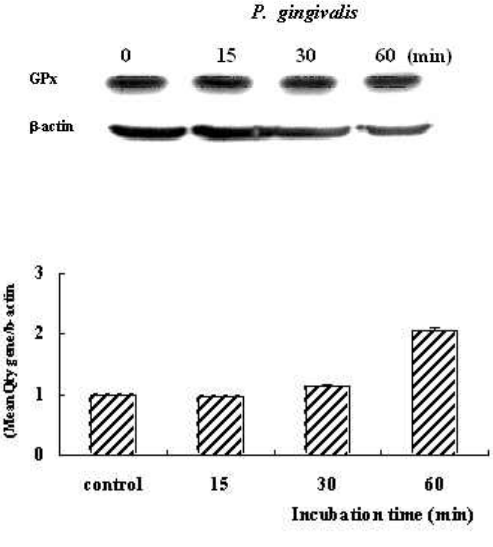

Figure 7. Expression of the GPx in KB cells infected with P. gingivalis. Western blot analysis of GPx expression in KB cells infected with P. gingivalis at an MOI of 100 for the time periods indicated. Intensities were quantified by densitometry with the intensity of uninfected control cells set to 1.

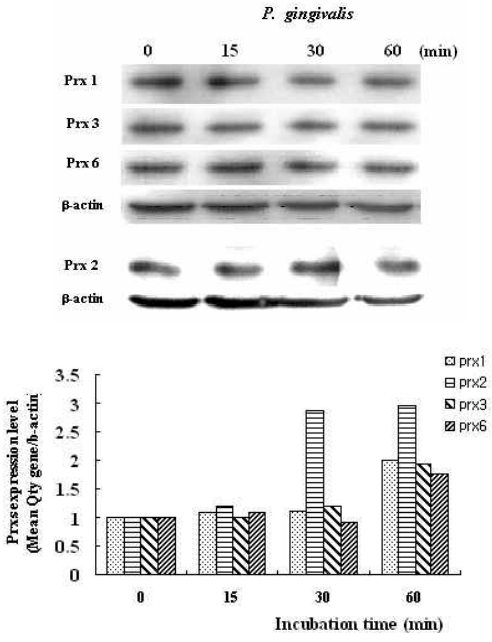

Figure 8. Expression of the peroxiredoxins (Prxs) in KB cells infected with P. gingivalis. Western blot analysis of Prx1, 2, 3, and 6 expression in KB cells with P. gingivalis at an MOI of 100 for the time periods indicated. Intensities were quantified by densitometry with the intensity of uninfected control cells set to 1.

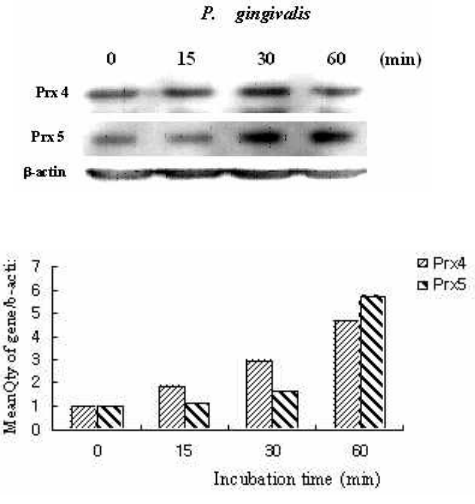

Figure 9. Expression of the Prx4 and Prx5 in KB cells infected with P. gingivalis. Western blot analysis of Prx4 and Prx5 expression in KB cells with P. gingivalis at an MOI of 100 for the time periods indicated. Intensities were quantified by densitometry with the intensity of uninfected control cells set to 1.

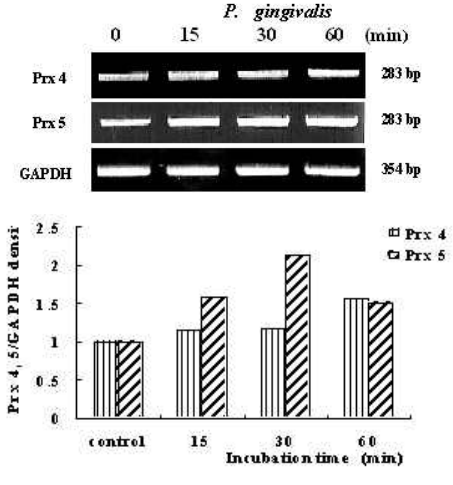

Figure 10. Expression of mRNA for Prx4 and 5 in KB cells infected with P. gingivalis. KB cells were infected with P. gingivalis (MOI 100) and incubated for the indicated time periods, and total RNA was prepared. The expression levels of PrxⅣ, PrxⅤ and GAPDH mRNA were measured using RT-PCR.

9. Induction of mRNA for PrxⅣ and PrxⅤ after P. gingivalis infection.

KB cells were infected with P. gingivalis for the different time periods (0~60 min). P. gingivalis stimulated PrxⅣ and PrxⅤ induction was measured by RT-PCR. As shown in Figure 10, PrxⅣ and PrxⅤ transcripts were time dependently increased.

Ⅳ. DISCUSSION

Porphyromonas gingivalis is an asaccharolytic, black- pigmented, gram-negative anaerobic bacterium. P. gingivalis is a major etiologic agent of chronic periodontitis, and may be

involved in systemic illness, such as atherosclerosis[19,20]. Adherence to and invasion of epithelial cells by P. gingivalis may play an important role in the pathogenesis of periodontal disease[21,22].

P. gingivalis fimbriae bind to cytokeratin of epithelial cells, and thus involved in adherence and invasion of P. gingivalis in epithelial cells [23]. There is a report describing the induction of defensin (one of cationic antimicrobial peptides) resistance in P. gingivalis infection[24]. Such resistance may have an effect on the ability of oral pathogens to persist in the mouth and to withstand innate human immunity.

Epithelial cells have been considered relatively passive cells which function uniquely to protect the body surfaces and migrate to cover exposed tissue during wound healing. When activated, epithelial cells can behave very aggressively by migrating, proliferating, and producing various cytokines, and proteolytic enzymes. Therefore they play an active role in inflammation and in defense against microbes. For the study of pathogenesis of P. gingivalis infection, the established KB cell line was used, whose proliferation rate and viability are constant. Most studies used P. gingivalis (ATCC 33277) as the ratio of 100 bacteria per cell in this study[21,22,25].

ROS notably superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide, operate on a variety of physiological processes, including host defense, gene expression, oxygen sensing, regulation of vascular tone, bone resorption, apoptosis, cell growth, and transformation[26]. NADPH oxidase plays a key role in ROS generation for host defense[27]. The catalytic component of NADPH oxidase is comprised of several proteins encoded by the NOX gene family. Several lines of evidence suggest that NOX regulates cellular responses to various growth factors[28]. In guinea pig gastric pit cells, NOX1 enhances O_2^- generation in response to Helicobacter pylori lipopolysaccharide (LPS), signaling through Toll-like receptor 4 (TLR4)[29]. This increased oxidase activity reflects the action of LPS on several targets, including activation of Rac1 and transcription of Nox1 and its organizer, NOXO1[13]. The rate of O_2^- generation by P. gingivalis treated KB cells accounts for only 0.5 and 5% of those by human neutrophils and macrophage colony-stimulating factor-activated mouse peritoneal macrophages[30], respectively. The amount is not enough to kill microorganisms directly, but it is enough to enhance proinflammatory cytokine production of the large intestinal epithelial cells[31]. Thus NOX1 may constitute the early responses of epithelial cells against pathogens as part of local host defense. Geiszt M et al[32]. suggested that NOX1 may be functionally similar to Nox2 and appears to replace NOX2 in the regulated production of O_2^-. Instead of using gastric mucosal cells, KB cells were used in this study.

P. gingivalis increased transcription of NOX4 rather than NOX1, but activation of Rac1 and NOXO1 was similar to the previous work[13]. At present, it is not known whether the P. gingivalis-dependent activation of the NOX1 expression actually participates in mucosal host defense or inflammation. It is now recognized that ectopic expression of Nox1 alone in cultured cells does not result in O_2^- production and that the coexpression of NOXO1 and NOXA1 is necessary for generation of a large amount of O_2^-. In fact, IL-1β and flagellin from

Salmonella enteritidis induce NOX1 in T84 cells, whereas both factors neither induce NOXO1 nor upregulate O_2^- production. In contrast, IFN-γ stimulates a small but significant induction of NOXO1 as well as NOX1[33].

Several lines of evidence implicate H_2O_2 as an intracellular messenger modulating the extent of protein phosphorylation[34,35]. The binding of growth factors to their receptors results in the activation of phosphatidylinositol 3-kinase, the products of which (PtdIns[3,4,5]P_3 and PtdIns[3,4]P_2) then bind to the pleckstrin homology domain of NOX-associated ß-Pix and stimulate the GDP-GTP exchange activity of ß-Pix. The activated ß-Pix converts Rac1-GDP to Rac1-GTP, which then associates with NOX1 to promote the electron transfer from NADPH to molecular oxygen[28]. NOX1 and NOX4 are also involved in angiotensin II-induced signaling in smooth muscle cells and insulin-dependent signaling in adipose tissues[27]. Overexpression of NOX and increased ROS levels are seen in cells and tissues with a strong mitogenic activity. NOX overexpression has been reported in human cancer cells, including colon cancer (NOX1)[9], melanomas (NOX4)[36], and prostate cancer (NOX4 and NOX5)[37].

NOX1 activity is regulated by accessory proteins such as NOXO1 and NADPH activator[18], whereas NOX4 is constitutively active and does not require accessory proteins. It has been shown that the regulation of NOX isozymes as well as their expression level plays an important role in cellular proliferation[38]. The level of NOX4-induced ROS generation is likely determined mainly by the expression level of NOX4 itself.

Superoxide dismutase (SOD) is one of the antioxidant enzymes in protecting the cells against the deleterious effects of ROS. In humans, three forms of SOD have been identified, each with distinct distribution and metal components copper/zinc-superoxide dismutase (Cu/Zn-SOD, SOD1), manganese superoxide dismutase (Mn-SOD, SOD2), and extracellular superoxide dismutase (EC-SOD, SOD3). They convert $O_2^{-\bullet}$ into H_2O_2, which is subsequently neutralized to oxygen and water by catalase and glutathione peroxidases[39,40]. They detoxify H_2O_2 into H2O and cooperate with other enzymatic and non-enzymatic antioxidants to regulate the intracellular levels of ROS[41]. The main functions of Prxs was to protect cells against oxidative attacks[42]. Up to date, at least six isotypes of Prxs (Ⅰ- Ⅵ) have been identified in mammals. Among the six mammalian Prxs, PrxsⅠ, Ⅱ, Ⅲ, Ⅳ and Ⅴ have been detected at least in rat heart, lung, pancreas, liver, and spleen homogenates[43-46] and Prx Ⅵ mRNA in rat lung and alveolar pneomocytes[47]. PrxI is a stress-inducible protein which is associated with cell signaling pathways[48,49]. Prxs participate in cellular antioxidant defense; some of them, such as PrxⅡ[50], also induce cell proliferation and protect cells from undergoing apoptosis. PrxⅣ also functions in the extracellular space[51]. Prx Ⅴ shows 10% sequence identity to PrxⅠ-Ⅳ, and is expressed at least in the fluid of bronchoalveolar lavage and lung homogenates[44,52]. PrxⅤ newly described mammalian cytosolic peroxiredoxin is subcellularly located to peroxisomes and mitochondria, places where protection againsts ROS is mainly required. PrxⅥ contains one cysteine, in contrast to the two cystein residues in other Prxs[15].

KB cells started their defense by increasing

the expression of SOD, GPx, Prxs after P. gingivalis infection. But intracellular oxygen radical production by KB cells endocytosing P. gingivalis (MOI 100) was not increased because this superoxide production by KB cells might be, in part, due to in size of the P. gingivalis. P. gingivalis are only 1μm in length[53]. From this study it can be hypothesized that P. gingivalis may potentially induce oxidative stress and consequent DNA damage, arrest of cells in S phase, P. gingivalis may induce notable GSH depletion and intracellular ROS production. However, additional studies are needed to extend this hypothesis.

V. CONCLUSION

Porphyromonas gingivalis has been implicated as one of the important bacterial species in pathogenesis of severe forms of periodontitis. The adhesion of P. gingivalis to host epithelium induces changes in both cell types as bacteria initiate infection and epithelial cells start their defense. It can be hypothesized that antioxidants involved in ROS scavenging have different distribution and expression in oral epithelial cells infected with P. gingivalis.

This study was aimed to investigate NADPH oxidase components in the generation of ROS and expression of antioxidant proteins in oral epithelial cells (KB cells) infected with P. gingivalis.

From this study it was suggested that superoxide radical productions through NADPH oxidase such as NOX4 and Rac1 was increased. NOX1 expression was regulated by NOXO1 and NOXA1 in response to P. gingivalis. The enzymes of antioxidative defense, like as Prx4 and Prx5, seemed to play a role in protecting P. gingivalis infection.

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Porphyromonas gingivalis 감염된 구강상피세포에서 NADPH oxidase와 항산화단백의 발현

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Porphyromonas gingivalis는 치주질환을 야기하는 독성세균으로서, 구강상피세포에 P. gingivalis가 감염되었을 때, 세포형태에 변화를 초래함으로 인해 방어기작이 작동하게 된다. 치주질환과 관련되어 생성된 활성산소종의 소거에 관여하는 항산화성분은 P. gingivalis 이 감염된 구강상피세포에서 그 분포와 발현수준이 달라지리라 예상된다.

따라서 이번 연구에서는 구강상피세포(KB 세포)에 P. gingivalis가 감염되었을 때 야기되는 활성산소종과 이를 소거하는 역할을 하는 항산화단백들의 역할들을 규명하고자 하였다.

활성산소종 형성을 조절하는 NADPH oxidase 중 NOX4와 Rac1 전사체는 구강상피세포에서 P. gingivalis 세균에 의해 증가하였으며 $gp91^{phox}$, Rac2, $p47^{phox}$ 와 $p67^{phox}$는 세균에 의한 변화가 관찰되지 않았다. 반면에 $p40^{phox}$ 전사체는 감소하는 경향을 보였다. NOX1 전사체는 P. gingivalis 처리 30분 후 감소하였다가 60분 후에는 다시 증가하는 양상을 보였다. 같은 기간에 NOX 활성화 단백인 NOXA1은 감소하고, NOX 구성단백질인 NOXO1은 증가하는 경향을 보였다.

P. gingivalis가 감염된 구강상피세포를 방어하는 항산화단백 발현수준을 평가한 결과, SOD1, 2, 3 모두 P. gingivalis 처리시간에 따라 증가하는 양상을 보였다. GPx 발현 양상도 SOD와 유사하게 나타났다. H_20_2를 소거하는 Prx는 감염된 KB 세포에서 Prx4와 Prx5가 4-6배 증가하는 것을 알 수 있었다. 반면 endocytosis 과정 중 H_20_2 생산은 변화되지 않았다.

이번 연구의 결과, P. gingivalis의 감염은 KB 세포의 NOX4와 Rac1의 NADPH oxidase 발현을 증가시켰으며, NOX1은 NOXA1과 NOXO1의 조절에 의해 영향을 받음을 알 수 있었다. 또한 항산화기작으로는 SOD, GPx, Prx가 증가하였는데, 이것은 Prx4와 Prx5가 중요한 역할을 할 것을 시사하였다.

주요어 : Porphyromonas gingivalis, KB cells, NADPH oxidase, antioxidant protein