The effect of cigarette smoking on *Porphyromonas gingivalis*, a crucial periodontal pathogen

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Smoking is implicated as one of the most significant risk factors for periodontal disease. The objective of this study was to examine the effect of cigarette smoke extract (CSE), on cultivation of and protein production by *Porphyromonas gingivalis*, a major periodontal pathogen. *P. gingivalis* were cultivated in the presence of CSE (0%, 2.5%, 5%, and 10%) solution, and their growth was measured by optical density. In addition, expression pattern of proteins produced by *P. gingivalis* under CSE influence, was examined by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). According to the results, the growth of *P. gingivalis* was inhibited by CSE in dose-dependent mode. SDS-PAGE analyses revealed minor difference in expression pattern of proteins. Results of this study suggest that CSE imposes an inhibitory influence on growth of *P. gingivalis*, and is capable of modulating protein expression in *P. gingivalis*.

Key Words: Microbiology, *Porphyromonas gingivalis*, Smoking, Virulence

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INTRODUCTION

Periodontal disease is a common inflammatory disease that can lead to destruction of supporting structures of the tooth and, finally tooth loss, if not controlled [1,2]. It has been generally accepted that dental plaque is initially responsible for periodontal disease. Dental plaque consists of more than several hundred different bacterial species, and has potential to dysregulate a homeostasis maintained by the host, leading to dysbiosis [3,4]. Host immuno-in-

flammatory activities in response to dental plaque through host-parasite interaction, can lead to clinical manifestations of periodontal disease. The result of this interaction could also be modulated, by other known risk factors or disease modifiers. These factors, either inherent or acquired, may considerably affect the natural history of different types of periodontal diseases [5-7].

Cigarette smoking is one of most important risk factors for numerous diseases, and so it has been implicated in initiation and progression of periodontal disease [8]. In the

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past several decades, abundant epidemiological data have demonstrated that smoking contributes to increased risk for periodontal disease [9,10]. However, exact pathological mechanisms by which smoking influences periodontal disease are not completely understood, although it was suggested that changes in immune and inflammatory responses may be partially responsible for periodontal disease in smokers [11].

Numerous data have shown that cigarette smoking has a negative effect on periodontal disease, as smokers were found with increased alveolar bone loss, tooth mobility, probing depth, and tooth loss than non-smokers [12]. In addition, smokers are less effective on surgical or non-surgical periodontal treatment, compared to non-smokers [13,14]. Although how smoking affects periodontal disease has not been elucidated, smoking may modulate host immune response and behavior of periodontal microorganisms in the host [15].

The relationship between smoking and dental plaque microorganisms has not been clearly elucidated. It was revealed that rate of plaque formation was not affected by smoking [16,17]. Conversely, some studies found that certain bacterial species, including *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* were more abundant in smokers [18-20], while others were unable to detect differences in microorganisms between smokers and non-smokers [16,21,22]. *P. gingivalis* is a gram-negative, asaccharolytic, non-spore-forming, anaerobic black-pigmented bacterium, and has been regarded as a crucial bacterial pathogen involved in initiation and progression of periodontal disease. It has been frequently recovered from the periodontal lesion, and found to possess potent virulence factors including fimbriae, hemagglutinins, a broad spectrum of proteases and other enzymes, lipopolysaccharides, and a capsule [23,24].

Bacterial pathogens are equipped with sophisticated mechanisms for adapting to complex environmental changes, and so, ensure adequate growth and survival within the host [25,26]. For example, iron and heme use by *P. gingivalis* adopt complex schemes, to ensure optimal nutrient uptake in response to environmental changes [27]. Also, expression of virulence factors of *P. gingivalis* is regulated as well. It was observed that the activity of the

fimbrial gene (*fimA*) decreased by approximately 50%, in response to hemin limitation [28]. Recently, it was found that cigarette smoke extract (CSE) can modulate expression of several virulence factors produced by *P. gingivalis* [29,30], and so smoking can alter humoral responses, and may increase *P. gingivalis* infectivity [31]. Collectively, results suggest that smoking, as an environmental risk factor, may affect behavior of *P. gingivalis*, leading to modification of host-parasite interaction.

The purpose of this study was to assess the effects of CSE on growth and overall protein expression of *P. gingivalis*.

MATERIALS AND METHODS

Bacterial strain and growth conditions

P. gingivalis 381 was purchased from ATCC (Manassas, VA, USA) and maintained on anaerobic blood agar plates at 37°C under anaerobic conditions (80% N₂, 10% CO₂, 10% H₂), in a Forma 1025 Anaerobic Chamber (Thermo, Marietta, OH, USA). For liquid growth, bacterial cells were cultured in 3% (w/v) trypticase soy broth supplemented with 0.5% yeast extract, 5 mg/L hemin and 1 mg/L menadione. *Escherichia coli* DH5 α was obtained from Promega (Madison, WI, USA) and grown in Luria-Bertani media, as described elsewhere. Growth rates of *P. gingivalis* 381 were determined by measuring optical density at 600 nm, using a spectrophotometer.

The growth of *P. gingivalis* 381 after exposure to CSE

Various CSE solutions were prepared using a specially designed apparatus as follows (Fig. 1). After a cigarette was connected to the apparatus and lit, cigarette smoke was sucked into a phosphate buffered solution (PBS) buffer by negative pressure. Commercial cigarettes (Marlboro Red; Philip Morris, Inc., Richmond, VA, USA) were used. A total of three cigarettes were used per 30 mL of PBS, to generate a CSE-PBS solution, and each cigarette was smoked for approximately 4 minutes and 30 seconds. *P. gingivalis* of 5×10^7 cells were added to 20 mL liquid broth, containing varying final CSE concentrations (i.e., 0%, 2.5%, 5%, and 10%) respectively, and cultivated for 48 hours. The effect of

Fig. 1. An apparatus used for the preparation of cigarette smoke extract solution. A cigarette smoke was sucked into a phosphate buffered solution-containing cylinder by negative pressure.

Fig. 2. The growth pattern of *Porphyromonas gingivalis* in the presence of cigarette smoke extract (0%, 2.5%, 5%, and 10%). The error bars indicate the range of data, as the experiments were performed in triplicates.

CSE on growth pattern and protein expression of *P. gingivalis* was measured.

Preparation of cell lysates and SDS-PAGE

Bacterial cell lysate was prepared by adopting a SMART Bacterial Protein Extraction Solution (iNtRON Biotech Inc., Seongnam, Korea), according to manufacturer's instructions. Conventional sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed according to standard protocols. Protein samples were solubilized in 0.0625 M Tris hydrochloride (pH 6.8) containing 3% sodium dodecyl sulfate, 20% glycerol, 0.005 M ethylenediaminetetraacetic acid, and 1% 2-mercapto-

Fig. 3. Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis analysis of total cellular lysates of *Porphyromonas gingivalis* after exposure to CSE (0%, 2.5%, 5%, and 10%); A molecular size marker was loaded in the left lane. A protein band (indicated by an asterisk mark) with molecular weight (MW) around 70 kDa was shown in proteins of *P. gingivalis* grown in 2.5%, 5%, and 10% CSE.

ethanol. Protease inhibitor (Sigma-Aldrich Co., Dorset, UK) was added, as required, directly to the first dimension sample buffer. Samples were then heated at 100°C for 5 minutes and applied to the gel. Electrophoresis was performed at room temperature, and at a constant voltage (100 V). Changes of protein expression in SDS-PAGE were evaluated by visual inspection using unaided eyes.

RESULTS

It was observed that growth of *P. gingivalis* 381 was inhibited after exposure to CSE for 48 hours (Fig. 2). Growth of *P. gingivalis* cells pre-exposed to CSE was inhibited at 24 hours, compared to normal growth without CSE. For the next 24 hours, *P. gingivalis* cells pre-exposed to 2.5% and 5% CSE revealed a similar pattern to that of growth without CSE. However, *P. gingivalis* exposed to 10% CSE exhibited a four-fold greater inhibition.

Total protein profiles of *P. gingivalis* after 48 hours of culture in the presence of CSE solutions, as shown by SDS-PAGE analysis in Fig. 3, exhibited minor difference at molecular weights of around 70 kDa. A protein band, indicated by asterisk mark, shown in 2.5%, 5%, and 10% CSE solutions, did not appear in a clear pattern in 0% CSE solutions.

DISCUSSION

Results of this study indicate that growth of *P. gingivalis* was inhibited in dose-dependent manner when exposed to CSE for 48 hours, as expected. In this study, 0% to 10% CSE were used, as CSE concentrations were determined that is equal to nicotine concentration in saliva and gingival crevicular fluid [32]. While growth was strongly inhibited in all CSE concentrations for the first 24 hours, bacterial cells cultured in the presence of 2.5% and 5% CSE did not reveal significant growth inhibition for the next 24 hours. Only cells cultured in 10% CSE solutions exhibited an inhibition after 48 hours. It is likely that *P. gingivalis* may have adapted to lower concentrations of CSE during the first 24 hour-growth, and overcome inhibitory effect of CSE, resulting in optimal bacterial growth at 48 hours. This finding is similar to that of our previous study using nicotine, where Baek et al. [33] found that *P. gingivalis* cells pre-exposed to nicotine revealed almost two-fold increase in growth rate, when cells were cultured again in the presence of nicotine. As the effect of smoking is most likely chronic for most individuals, it is possible that continuous exposure to smoking will enhance the capability of bacterial cells to handle subsequent exposures to smoking. So, smoking may lead to increased potential of *P. gingivalis* virulence.

Bacteria adapt to environmental changes for their survival. It has been demonstrated that expression of *P. gingivalis* virulence factors was environmentally modulated [34-40]. It was also observed that this microorganism can adapt to smoking, by alternating gene expression. Bagaitkar et al. [29] revealed that *fimA* was upregulated with differential regulation of 6.8% of *P. gingivalis* genes, and that decreased inflammatory response was induced by CSE.

In this study, it was expected that CSE, as complex toxic substances [41,42], could induce modulation of growth and virulence of *P. gingivalis* that can lead to production of stress-related proteins. However, results revealed that CSE do not significantly affect total protein expression, as observed by SDS-PAGE (Fig. 3), exhibiting changes only in expression of proteins with molecular weight, approximately 70 kDa. It was rather surprising that there was no major difference in protein expression of *P. gingivalis* after exposure to CSE. Results suggest that CSE can affect only

selected groups of proteins. However, it cannot be ruled out that significant change in the protein profile may have occurred, and was not detected by visual inspection using one-dimensional gel electrophoresis analysis. Compared to a previous study using only nicotine [33], this study has used CSE containing approximately 7,000 tobacco substances, in addition to nicotine [41,42]. So, it was expected to yield more reliable information regarding the effect of smoking on growth, and protein expression of *P. gingivalis* using CSE.

Considering limitations of this study, it can be concluded that CSE has potential to elicit stress reaction, and so may serve as an environmental modulating factor, for bacterial growth and survival. Future studies using more sophisticated technology such as two-dimensional gel electrophoresis and proteomics analyses, may reveal important information regarding virulence-related proteins of *P. gingivalis* that may influence host-parasite interactions in the host.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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