Cytotoxic Effects of *Prevotella nigrescens* on Cultured Cells

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국문초록

*Prevotella nigrescens*가 배양된 세포에 미치는 영향

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흑색 색소를 형성하는 그람음성 혐기성 세균은 급성 임상 증상을 가진 환자의 근관에서 자주 발견되는 세균으로서 세균 및 세균의 성분과 산물이 치근단 병소의 생성과 밀접하게 연관된 것으로 알려져 있다.

본 연구는 흑색 색소를 형성하는 그람음성 혐기성 세균 중 가장 발현율이 높은 *Prevotella nigrescens*가 배양된 세포에 미치는 세포 독성을 연구하고자 하였다. 두 가지 세포주 및 사람의 치은섬유모세포를 일차배양하여 사용하였으며, 세포주에 따른 독성 발현에 차이가 있는지를 비교하였다. *P. nigrescens* ATCC 33563 표준 균주 및 임상 균주로는 환자의 감염된 근관으로부터 16S rRNA primer를 사용한 중합효소 연쇄반응으로 *P. nigrescens* 6 균주를 동정하여 사용하였다. 세균배양액, 세균의 초음파 추출단백질 및 lipopolysaccharide (LPS)를 MC3T3-E1 조골세포, NIH3T3 섬유모세포 및 치은섬유모세포에 첨가한 후 MTT분석법으로 세포의 활성을 측정하였으며, 세포의 형태학적 변화를 도립현미경으로 관찰하였다.

세균배양액을 100㎕ 첨가한 경우는 세가지 세포주 모두에서 통계적으로 유의하게 세포의 활성을 억제하였다. 세균의 초음파 추출단백질 12.5㎍/㎖ 와 25㎍/㎖ 는 NIH3T3세포에 통계적으로 유의한 세포독성을 보였다. 세 가지 세포주에 대한 LPS의 세포 독성 효과는 첨가된 LPS의 농도 및 균주에 따라 다양하게 나타났다. 심하게 손상된 세포는 세포의 단일층이 수축되고 세포가 응집되었으며 세포가 배양용기의 바닥에서 떨어지는 양상이 도립 현미경하에서 관찰되었다. 본 연구의 결과 *P. nigrescens*가 숙주 반응을 조절하여 치수 및 치근단 병소의 유발 및 악화에 기여하는 세균으로 작용할 수 있음을 시사한다.

Ⅰ. INTRODUCTION

Infection of the dental pulp commonly occurs as a consequence of caries. Pulpal infections often progress to pulp necrosis and periapical lesion development with periapical bone destruction. The severity of pulpal/periapical inflammation has been directly correlated with the total microbial content within root canals, and with the length of time the periapical tissues were exposed to the infecting microorganisms[1]. Microbial studies of endodontic infections have revealed that gram-negative anaerobic bacteria are the prominent microorganisms[2,3]. Some combinations of bacterial species present in root canals are more pathogenic in inducing periapical inflammation and bone destruction[4,5]. Species of *Prevotella*, *Porphyromonas*, *Fusobacterium*, and *Peptostreptococcus* are strongly linked to increased periapical destruction[3]. Sunqvist et al[5] identified 25 strains of black pigmented *Bacteroides* in 22 of 72 infected root canals and periapical abscesses. The most common iso-

late was *Bacteroides intermedia*, consisting of 14 strains of 25 isolated strains. Van Winkelhoff et al[6] isolated one or more species of *Prevotella* and *Porphyromonas* in all abscesses of endodontic origin. *P. intermedia* was the most frequently isolated strain comprising 63% of the examined abscesses.

Many of the pathogenic effects of the endodontic infections are due to bacterial by-products in addition to the effects of these products on the host-derived soluble mediators such as cytokines, rather than the direct necrotizing effects of the bacteria on the tissues[7]. Soluble bacterial components, including cell wall structure, lipopolysaccaride (LPS) and toxins result in the stimulation of specific immune responses characterized by the activation of T- and B-lymphocytes[8]. Polymorphonuclear leukocytes are attracted to site of infection by a number of bacteria- derived chemoattractant[9].

The species *Prevotella intermedia* have been subdivided into the species *Prevotella intermedia* and *Prevotella nigrescens* according to the taxonomic changes[10]. Four out of fifteen isolates (27%) were re-identified as *P. nigrescens*, using biochemical methods and SDS-PAGE of whole cell protein analysis tentatively identified as *P. intermedia* in endodontic infection[11]. Baumgartner et al[12] used conventional biochemical methods and molecular biologic methods, using polymerase chain reaction of specific primers for 16S rRNA, to differentiate *P. nigrescens* from *P. intermedia* collected from infected root canals. 11 of 22 (50%) of were identifed as *P. nigrescens* and 8 of 22 (36%) were *P. intermedia*.

Cytotoxicity of some Gram-negative bacterial species was studied. *P. gingivalis* ATCC 33277 was strongly cytotoxic to gingival fibroblasts[13]. Sonicated bacterial extracts (SBEs) from anaerobic Gram-negative bacteria inhibited the growth of the periapical fibroblasts[14]. *In vitro* testing of cytotoxicity using cell culture has the advantage of controllability, reproducibility, and rapidity compared to *in vivo* animal study. However, there is evidence that different cell types display varying sensitivities to different test materials[15]. The cytotoxicity of growth supernatants from some bacterial species was specific against cells of various origin and from different individuals[13].

One approach to elucidate the role of the black-pigmented bacteria most commonly identifed in infected root canals as developing and progressing pulp/ periapcial lesions would be to investigate the toxic effects of metabolic products, cell proteins, and LPS of those bacteria. There has been no study on the cytotoxicity of both *P. nigrescens* ATCC type strain and clincial isolates of *P. nigrescens* from infected root canals.

The purpose of this study was to investigate the toxic effects of *P. nigrescens* ATCC type strain and *P. nigrescens* isolated from infected root canals having acute signs and symptoms. Cytotoxic effects of bacterial growth medium supernatants, sonicated bacteiral extracts and LPS were analyzed. Two cell lines, MC3T3-E1 and NIH3T3, and human gingival fibroblast were used to compare the possible specific sensitivity of their cytotoxicity against cell types.

Ⅱ. MATERIALS AND METHODS

1. Bacterial Sampling Procedure

The bacteria had been isolated from the root canals of patients experiencing acute clinical symptoms who were treated at the Dental Clinic at Wonkwang University, College of Dentistry. Patient's symptoms of spontaneous pain, tenderness to percussion, and swelling were recorded. Patients who had taken antibiotic medication within the preceding 3 months were excluded from this study. Briefly, the tooth was isolated using a rubber dam and disinfected. After access cavity was opened, sterile paper points were introduced into the canal and then placed in 1 ml of reduced transfer fluid.

2. DNA Extraction and PCR Amplification for Identification of *P. nigrescens*

Pure cultures of bacteria from blood agar plates were collected into a microcentrifuge tube and

washed three times with distilled water. Cells were centrifuged for 10min at 7,500rpm and supernatant was discarded. Genomic DNA was isolated using a DNeasyTM Tissue Kit (Qiagen, Germany) The resulting pellets were stored at -20 ℃ before PCR amplification. PCR oligonucleotide primers were developed from published data[16] for the 16S rRNA gene of *P. nigrescens* ATCC 33563 (sense primer: GTGTTTCATTGACGGCATC-CGATATAGAAC, antisense primer: CCACGTC-TCTGTGGGCTGCGA). Cycling conditions were at 94℃ for 5min, 30 cycles at 94℃ for 1min, 65℃ for 1min, and 72℃ for 1min; and ending with 72℃ for 5min. PCR products of ~828 bps were confirmed by 1.2% agarose gel electrophoresis, visaulized by ethidium bromide fluorescence and photographed. Amplicon size was analyzed by comparison to a 1kb DNA ladder (Promega, Madison, USA).

3. Bacterial Growth Conditions

Six bacteria isolated and identified as *P. nigrescens* from teeth with acute symptoms and *P. nigrescens* ATCC type strain were included. These bacteria were grown in BM broth containing 1% tryptone (Difco, Detroit, MI, USA), 1% proteose peptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, 5㎍/ml of hemin, and 0.5㎍/ml of menadione in an anaerobic chamber (N_2, 90%; H_2, 5%; CO_2, 5%, Sheldon Manufacturing Inc. USA) at 37℃.

4. Bacterial Growth Medium Supernatants

Bacteria from blood agar plates were grown in BM broth for 72 hrs at 37℃ in an anaerobic condition. The optical density (OD) of bacteria was measured at 650nm. 500㎕ (OD 1.0) of each bacterial suspension was added to tubes containing 3 ml BM medium and allowed to grow for 72hrs at 37℃. The bacterial suspensions were centrifuged at 3000rpm for 15min. The supernatant fluids were filter-sterilized through 0.22-㎛ membranes (Milipore, USA). Supernatants were immediately frozen, stored at -80℃ before they were used in cytotoxicity tests.

5. Sonicated Bacterial Extracts

The bacterial cells were harvested by centrifugation (3,000rpm) for 30min then washed three times with PBS. The concentrated cell suspensions were sonicated thirty times in an ice box with bursts from a sonifier (Bio-Rad, USA) for 1 min to prevent heat generation, and the supernatants were then recovered. The SBE preparation was sterilized with a syringe filter (0.22㎛). The protein contents of SBEs were determined by the Bradford Protein Assay (Bio-Rad Laboratories, USA). The SBE was diluted with PBS to a final concentration of 2.5㎎ of protein/ml and stored at -80℃ until used.

6. LPS Purification

LPS was extracted from 1g wet weight of *Prevotella nigrescens* (ATCC 33536) and clinical isolates using the Eidhin and Mouton method[17]. Broth cultures were harvested by centrifugation and washed in 1/10 volume of a wash buffer (20mM Tris-HCl, pH 7.4, 0.15M NaCl, 10mM $MgCl_2$) to remove any residual medium. The bacteria were then reharvested, lyophilized and ground to a powder in a mortar. Twenty-five milligrammes of powder were placed in a microcentrifuge tube, and mixed with 1ml of deionised water. The tube was then placed in a boiling water bath for 15min, with vortexing at 5-min intervals. The cellular debris was then collected by centrifugation at 12,000×g for 5min, and the supernatant removed to a fresh tube. Proteinase K (1mg dissloved in 50㎕ water) was then added, and the tube incubated for 1hr at 60℃. The tube was then placed in boiling water for 5min to precipitate any residual proteinase K and centrifuged as before. The supernatant was dialysed against water overnight, subjected to a further round of precipitation and centrifugation, and the resulting supernatant lyophilised. The final powder was weighed in order to calculate the yield of crude LPS and redissolved to a final crude LPS concen-

tratrion of 5mg/ml in phosphate buffered saline (PBS).

7. Electrophoretic Methods of LPS

To confirm the purity of LPS preparations, samples were subjected to sodium dodecyl sulfate polyacrylamide gel electophoresis (SDS-PAGE) with Tris-buffer using 12% gels. LPS samples suspended in PBS were heated in SDS sample buffer at 100℃ for 5min. Gel electrophoresis was carried out at 90-100V through the resolving gel. The current was stopped when the dye front reached the bottom of the gel. Gels were silver stained to confirm the presence of step-ladder-like LPS bands on the gels using Bio-Rad silver stain kit (Bio-Rad Laboratories, CA, USA).

8. Cell Culture

Human gingival fibroblast was obtained from a patient undergoing elective periodontal surgery. Clinically, the healthy tissue appeared firm, non-erythmatous, non-edematous, and non-bleeding. The tissue section was washed several times with a complete culture medium α-MEM (Gibco, Grand Island, NY), 10% fetal bovine serum (Gibco) supplemented with penicillin G sodium (100 units/㎖), sreptomycin sulfate (100㎍/㎖), and amphotericin B (0.25㎍/㎖). Gingival tissue was minced with a blade into small pieces and transferred into a 60mm^2 culture dish (Nunc, Roskilde, Denmark). Cultures were maintained at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5min, and aliquots of separated cells were subcultured. Cell cultures between the third and eighth passages were used in this study.

MC3T3-E1 osteoblast from mouse calvaria and mouse fibroblast (NIH3T3) were used as cell lines. NIH3T3 and MC3T3-E1 were grown in RPMI1640 (Gibco, Grand Island, NY) and α-MEM respectively. Other culture conditions were the same as human gingival fibroblast culture.

9. Cytotoxicity Assay

MTT assay was used to determine cell viability of three cell types exposed to each bacterial preparation. 2×10^5 cells were seeded in a 96-well tissue culture plate in 100㎕ of complete culture medium and allowed to attach for 24hr in a 5% CO_2 incubator at 37℃. The cells were washed twice with phosphate-buffered saline. Cells were then exposed to various concentrations of each bacterial preparation for 24hrs. The volume of bacterial supernatants was 20㎕ and 100㎕. The same volume of BM broth was used as the control group. The amount of SBE tested was 12.5㎍/㎖ and 25㎍/㎖. The same amount of PBS was added in the control group. 1.5mg/㎖ and 2.5mg/㎖ of LPS was added in the experimental groups and the same amount of PBS was used as control. Five wells were used in each group. Cells were exposed to 50㎕ of MTT solution (2mg/㎖, Sigma) for 4 hours in a CO_2 incubator. The blue formazan precipitate was extracted using 100㎕ of dimethyl sulfoxide on a shaker at room temperature for 30 min. The absorption at 540nm (OD540) was determined using an ELISA reader (Spectra MAX 2500, Molecular Devices, USA). Results of the cytotoxicity experiments were expressed as a percentage of control tissues. Each measurement was performed with five replicates. Changes in cell morphology and detachment from the underlying surface were examined by inverted light microscopy at 400 x magnification.

19. Statistical Analysis

The significance of difference between the control and experimental groups was statistically analyzed by one-way ANOVA with the value of statistical sigificance at $p< 0.05$. The significance of difference among three cell types was also anlayzed. Tukey's HSD post-hoc test was used to determine significant differences between group means.

Ⅲ. RESULTS

1. Identification of *P. nigrescens*

Fig. 1 depicts amplicons from PCR amplification results for colonies of *P. nigrescens*. Primers of 16S rRNA for *P. nigrescens* yielded a product of 828-bp.

2. Electrophoresis of LPS

Silver-stained SDS-PAGE analysis of LPS prepared from ATCC type strain and clinical isolates revealed a typical LPS step-ladder like banding pattern with minimal protein (Fig. 2).

3. Cytotoxic effects of bacterial preparations compared to control using MTT assay

Cytotoxicity of bacterial preparations from *P. nigrescens* ATCC strain and clinical isolates exposed to three cell types was compared to the control groups. The addition of 20㎕ of BGS to all strains used in this study significantly increased cell viability of MC3T3-E1 osteoblasts ($p<0.05$, Table 1). However, the BGS from all strains was significantly ($p<0.05$) inhibitory at a dosage of 20 ㎕ on NIH3T3 (Table 2). Cell viability of human gingival fibroblasts was stimulated at the dosage of 20㎕ BGS of six strains except clinical isolate 2202 (Table 3). The BGS from all strains significantly ($p<0.05$) inhibited cell viability of three cell types at a dosage of 100㎕ (Table 1-3).

The SBEs from all strains had no statistically significant cytotoxic effect on MC3T3-E1 at a dosage of 12.5㎍/㎖. The SBEs from clinical isolates 304, 305, 2202, and 2302 were significantly ($p<0.05$) inhibitory on these cells at a dosage of 25㎍/㎖ (Table 4). The SBEs from all strains showed statistically significant cytotoxic effect on NIH3T3 at both dosages of 12.5㎍/㎖ and 25㎍/㎖ (Table 5). There was no cytotoxic effect on human gingival fibroblasts at the dosage of 12.5㎍/㎖ of SBE. The SBEs from all strains except clinical isolate 203 significantly ($p<0.05$) inhibited cell viability of human gingival fibroblasts at the dosage of 25㎍/㎖ (Table 6).

The LPS from *P. nigrescens* ATCC type strain significantly ($p<0.05$) inhibited cell viability of MC3T3-E1 at a dosage of 1.5㎎/㎖. LPS from clinical isolates exhibited no effect on these cells (Table 7). LPS from clinical isolates 203, 305, 2202, and 2302 was significantly inhibitory on NIH3T3 at the dosage of 1.5㎎/㎖. The LPS from *P. nigrescens* ATCC type strain stimulated cell viability of NIH3T3 at the dosage of 1.5㎎/㎖. The LPS from *P. nigrescens* ATCC type strain , clinical isolates 203, 305, and 2302 significantly inhibited cell viability of NIH3T3 at a dosage of 2.5㎎/㎖

Table 1. Cytotoxicity of *P.nigrescens* bacterial growth medium supernatants (BGS) to MC3T3-E1 osteoblast using MTT assay (% of control)

P. nigrescens	BGS volume	
	20㎕	100㎕
ATCC 33563	128.08±3.58*	68.08±2.20*
Clinical isolate 203	123.31±1.82*	66.23±4.08*
Clinical isolate 304	120.23±0.06*	66.51±1.42*
Clinical isolate 305	121.08±1.26*	74.50±1.41*
Clinical isolate 2202	118.38±0.44*	67.19±1.34*
Clinical isolate 2203	120.23±2.45*	69.77±1.19*
Clinical isolate 2302	122.31±0.63*	70.92±0.38*

Results are expressed as mean ± SD.
* $p<0.05$ compared to control (100%)

Table 2. Cytotoxicity of *P.nigrescens* bacterial growth medium supernatants (BGS) to NIH3T3 fibroblast using MTT assay (% of control)

P. nigrescens	BGS volume	
	20㎕	100㎕
ATCC 33563	87.04±1.17*	49.35±2.64*
Clinical isolate 203	90.80±2.34*	64.82±1.46*
Clinical isolate 304	85.06±2.67*	54.02±1.96*
Clinical isolate 305	89.10±0.84*	63.66±3.23*
Clinical isolate 2202	86.70±2.38*	63.27±2.21*
Clinical isolate 2203	88.91±3.54*	64.26±3.21*
Clinical isolate 2302	84.62±1.41*	66.70±2.03*

Results are expressed as mean ± SD.
* $p<0.05$ compared to control (100%)

($p<0.05$, Table 8). The addition of 2.5㎎/㎖ of LPS from clinical isolate 2203 showed a stimulatory effect on NIH3T3. The LPS from clinical isolates 2203 and 2302 showed stimulatory effects on human gingival fibroblast at the dosage of 2.5㎎/㎖. The LPS from other five strains had no effect on human gingival fibroblast at the dosage of 2.5 ㎎/㎖. The LPS from clinical isolate 2302 had a strong cytotoxic effect on human gingival fibroblast at the dosage of 2.5 ㎎/㎖ ($p<0.05$, Table 9).

4. Comparison of cytotoxic effects among cell lines

BGSs from seven bacteria had a significantly strong cytotoxic effect on NIH3T3 compared to MC3T3-E1 and human gingival fibroblast at the dosage of 20㎕ ($p<0.05$, Table 1-3). When compared to BGSs from clinical isolates 304, 305, and 2202, NIH3T3, MC3T3-E1, and human gingival fibroblast showed a significantly different sensitivity of cytotoxicity in descending order at the dosage of 100㎕ ($p<0.05$, Table 1-3).

SBEs from *P. nigrescens* ATCC type strain and all

Table 3. Cytotoxicity of *P.nigrescens* bacterial growth medium supernatants (BGS) to human gingival fibroblast using MTT assay (% of control)

P. nigrescens	BGS volume	
	20㎕	100㎕
ATCC 33563	123.87±9.95*	71.28±3.22*
Clinical isolate 203	124.47±5.15*	78.72±5.33*
Clinical isolate 304	124.07±4.26*	86.17±6.26*
Clinical isolate 305	122.93±5.28*	85.25±1.28*
Clinical isolate 2202	112.47±1.64	77.94±2.01*
Clinical isolate 2203	123.40±6.33*	78.30±3.51*
Clinical isolate 2302	122.53±7.35*	89.72±4.53*

Results are expressed as mean ± SD.
* $p<0.05$ compared to control (100%)

Table 4. Cytotoxicity of *P.nigrescens* of soinicated bacterial extracts(SBE) to MC3T3-E1 osteoblast using MTT assay (% of control)

P. nigrescens	SBE concentration	
	12.5㎍/㎖	25㎍/㎖
ATCC 33563	109.72±3.78	88.79±11.42
Clinical isolate 203	109.03±7.96	90.77±4.24
Clinical isolate 304	105.48±4.48	80.74±4.40*
Clinical isolate 305	112.42±6.21	74.80±1.62*
Clinical isolate 2202	103.79±14.29	58.05±3.16*
Clinical isolate 2203	116.82±12.70	88.52±1.68
Clinical isolate 2302	114.27±6.58	81.46±7.85*

Results are expressed as mean ± SD.
* $p<0.05$ compared to control (100%)

Table 5. Cytotoxicity of *P.nigrescens* of soinicated bacterial extracts(SBE) to NIH3T3 fibroblast using MTT assay (% of control)

P. nigrescens	SBE concentration	
	12.5㎍/㎖	25㎍/㎖
ATCC 33563	89.35±0.73*	82.49±2.23*
Clinical isolate 203	93.12±2.12*	89.38±2.21*
Clinical isolate 304	92.20±2.10*	85.72±2.98*
Clinical isolate 305	92.97±2.60*	89.21±2.00*
Clinical isolate 2202	91.40±1.88*	87.88±1.64*
Clinical isolate 2203	92.09±2.34*	89.59±2.97*
Clinical isolate 2302	91.29±1.22*	81.69±2.36*

Results are expressed as mean ± SD.
* $p<0.05$ compared to control (100%)

Table 6. Cytotoxicity of *P.nigrescens* of soinicated bacterial extracts(SBE) to human gingival fibroblast using MTT assay (% of control)

P. nigrescens	SBE concentration	
	12.5㎍/㎖	25㎍/㎖
ATCC 33563	113.25±12.28	86.31±4.45*
Clinical isolate 203	117.36±6.17	92.61±6.94
Clinical isolate 304	102.45±3.62	61.98±3.37*
Clinical isolate 305	106.65±3.80	65.47±2.85*
Clinical isolate 2202	109.02±3.04	62.54±5.22*
Clinical isolate 2203	120.03±14.85*	79.34±5.77*
Clinical isolate 2302	104.59±4.52	63.20±0.86*

Results are expressed as mean ± SD.
* $p<0.05$ compared to control (100%)

Table 7. Cytotoxicity of *P.nigrescens* LPS to MC3T3-E1 osteoblast using MTT assay (% of control)

P. nigrescens	LPS concentration	
	1.5㎎/㎖	2.5㎎/㎖
ATCC 33563	80.81±5.59*	70.23±11.79*
Clinical isolate 203	91.77±11.86	82.20±4.99*
Clinical isolate 304	109.92±0.31	86.82±7.09
Clinical isolate 305	111.21±6.91	77.73±6.31*
Clinical isolate 2202	122.18±13.07*	78.03±4.41*
Clinical isolate 2203	112.02±7.36	85.15±4.42
Clinical isolate 2302	85.16±2.79	14.47±12.80*

Results are expressed as mean ± SD.
* $p<0.05$ compared to control (100%)

Table 8. Cytotoxicity of *P.nigrescens* LPS to NIH3T3 fibroblast using MTT assay (% of control)

P. nigrescens	LPS concentration	
	1.5㎎/㎖	2.5㎎/㎖
ATCC 33563	116.03±8.68*	85.84±7.95*
Clinical isolate 203	79.34±2.44*	69.94±5.81*
Clinical isolate 304	91.45±3.20	92.66±5.97
Clinical isolate 305	83.95±1.80*	83.70±2.37*
Clinical isolate 2202	84.70±1.38*	91.59±6.71
Clinical isolate 2203	97.17±11.75	118.60±3.94*
Clinical isolate 2302	0.39±0.15*	14.45±7.77*

Results are expressed as mean ± SD.
* $p<0.05$ compared to control (100%)

Table 9. Cytotoxicity of *P.nigrescens* LPS to human gingival fibroblast using MTT assay (% of control)

P. nigrescens	LPS concentration	
	1.5㎎/㎖	2.5㎎/㎖
ATCC 33563	95.81±2.78	103.18±10.35
Clinical isolate 203	100.76±7.79	92.44±5.38
Clinical isolate 304	115.41±11.50	102.46±1.76
Clinical isolate 305	103.60±3.75	113.31±9.86
Clinical isolate 2202	105.47±10.22	106.38±15.02
Clinical isolate 2203	135.76±4.04*	112.73±10.53
Clinical isolate 2302	130.29±7.87*	19.88±5.57*

Results are expressed as mean ± SD.
* $p<0.05$ compared to control (100%)

of clinical isolates were inhibitory only on NIH3T3 cells at the dosage of 12.5㎍/㎖, showing that those cells were the most sensitive cell type compared to MC3T3-E1 and human gingival fibroblast ($p<0.05$, Table 4-6).

LPSs from seven strains had no cytotoxic effect on human gingival fibroblasts at both dosages of 1.5㎍/㎖ and 2.5㎍/㎖ except clinical isolate 2302. On the contrary, cell viability of MC3T3-E1 and NIH3T3 was affected by several strains of *P. nigrescens* at dosages of 1.5㎎/㎖ and 2.5㎎/㎖ showing that gingival fibroblast was the least sensitive in expression of cytotoxicity (Table 7-9).

5. Morphological changes under inverted microscopy

MC3T3-E1 osteoblasts exposed to 100㎕ of BGS from *P. nigrescens* ATCC type strain show morphological alteration. Cells exhibited rounded shape and granulations in the cytoplasm (Fig. 4).

NIH 3T3 fibroblasts exposed to 100㎕ of BGS from *P. nigrescens* ATCC type strain lost intercellular connection. Cells did not maintain confluent monolayer showing rounded cellular aggregations (Fig. 6).

Human gingival fibroblasts exposed to 2.5㎎/㎖ of LPS from clinical isolate 2302 completely lost normal spindle-shaped morphology. Cellular aggregations were observed, and these cells tended to float into the medium (Fig. 8).

Ⅳ. DISCUSSION

Bacterial infection of the pulp is the main etiologic agent of periapical lesion formation with the resorption of bone. Gram-negative black-pigmented anaerobic bacteria are the predominant microorganisms associated with the acute signs and symptoms of periapical lesions[16]. *P. nigrescens* was reclassified from *P. intermdia* species due to the differing results of multilocus enzyme electrophoretic analysis, DNA analysis, and SDS-PAGE analysis of soluble cellular proteins[19]. Bae

et al[20] reported that 41 (73.2%) were identified as *P. nigrescens* and 15 (26.8%) as *P. intermedia* of the 56 strains of black pigmented Bacteroides (BPB) isolated from endodontic infections. This result suggested that *P. nigrescens* was the most frequently isolated BPB from infections of endodontic origin.

Van Steenbergen et al[21] observed the filtered culture extracts from *B. gingivalis* and *B. asaccharolyticus* were toxic to Vero cells. Shah et al[22] showed the culture supernatant from low speed centrifugation of *P. gingivalis* culture was cytotoxic on human epithelial cell lines due to the presence of the cysteine proteinase in the supernatant. The BGS from *P. nigrescens* ATCC type strain and clinical isolates were cytotoxic to all three types of cells tested in this study at a dosage of 100㎕. Sensitivity difference among cell types were not observed in that concentration. On the contrary, the addition of 20㎕ of BGS stimulated cell viability of MC3T3-E1 and human gingival fibroblast, and there was no morphological alterations under the microscopy in those two cell types. These findings of stimulatory effects at a low dosage are consistent with other investigations[23,24]. Cytotoxicity was observed, however, in NIH3T3 culture at a dosage of 20㎕, although the inhibitory effect was weaker than at a dosage of 100㎕. This data indicates that NIH3T3 cells are more sensitive than other two cell types suggesting this cell type might be used as an early indicator of cytotoxiciy expression for BGS. The BGS contains all meabolic by-products of bacteria. It would seem that their end products are a direct cause of the cytotoxic effect on cells. These cells possibly are affected by the relatively low pH of BGS due to the breakdown ability of sucrose by saccharolytic *Prevotella*, as well.

The cytotoxicity of SBE was investigated using *P. gingivalis* and target cell was gingival fibroblast[24,25]. *P. gingivalis* displayed strong cytotoxic potential causing inhibition of fibroblast growth and morphological changes. Yamasaki et al[14] showed the SBE from *P. intermedia* ATCC 25611 had no cytotoxic effect on periapical fibroblasts at the dosages of 10㎍/㎖, 20㎍/㎖, and 30㎍/㎖, meaning *P. intermedia* was less cytotoxic compared with *P. endodontalis*, *P. gingivalis* and *F. nucleatum*. Cell viability of MC3T3-E1 and gingival fibroblast was not affected by 12. 5㎍/㎖ of SBE from *P. nigrescens* in this study. The cells did not show any morphological change. Cytotoxicity on MC3T3-E1 and gingival fibroblasts was observed at a dosage of 25㎍/㎖ of SBE. However, cytotoxicity of NIH3T3 was observed at both dosages of 12.5㎍/㎖ and 25㎍/㎖, although there was no significant difference between two dosages. This results are similar to the cytotoxicity of BGS on NIH3T3. It seems that NIH3T3 cells were more sensitive to the toxic effects of BGS and SBE than two other cell types.

Lipopolysaccharides (LPS) can stimulate osteoclastic resorption with relatively low potency[26]. The detection rate of LPS was higher in symptomatic teeth or teeth with radiolucent areas than in those without them[27]. LPS, purified from several gram-negative bacteria isolated from infected root canals, increased the rate of consumption of the C3 component of the complement[28]. LPS can activate B-lymphocytes to secrete antibody of diverse specificity[29]. LPS also acts as a potent stimulator of macrophages, inducing them to produce bone-resorptive cytokine, interleukin-1 and tumor necrosis factor[30]. Other cell wall components of gram negative bacteria such as a lipoprotein[31], muramyl dipeptide, a fragment of peptidoglycan[32], and lipid A associated protein[33] have been shown to have some biological effects similar to LPS. Human gingival fibroblasts express low levels of interleukin (IL)-6 constitutively and increased levels after stimulation with LPS34). In our experiment MC3T3-E1 cells exposed to 1.5㎎/㎖ of LPS from ATCC type strain showed a cytotoxic effect. The addition of 2.5㎎/㎖ of LPS from type strain, clnical isolates 203, 305, 2202, and 2302 inhibited cell viability of MC3T3-E1. On the other hand, cytotoxicity on NIH3T3 cells was observed at dosages of 1.5㎎/㎖ and 2.5㎎/㎖ from ATCC type strain, clinical isolates 203, 305, and 2302. The cytotoxicity on gingival fibroblasts was shown at a dosage of 2.5㎎/㎖ of LPS from clinical isolate 2302. LPS caused less cytotoxic

effects on three types of cells compared to BGS and SBE at those concentration used in this study. There were discrepances of cytotoxicity on three types of cells among the 7 tested *P. nigrescens*. LPS from clinical isolate 2302 had strong cytotoxicity on three types of cells compared to ATCC type strain and 5 isolates, which was not observed on those cells with the addition of BGS and SBE. Johansson et al[35] observed the interstrain discrepancies of cytotoxic effects on human gingival fibroblasts between 3 strains of *P. gingivalis*, 33277, 381, and W50. They suggested that these discrepancies might be an effect of the genetic variation between serotypes. Further study on the serotypes of *P. nigrescens* could explain the differences in cytotoxicity according to the strains.

The results from microscopic observation of damaged cells indicated that BGS, SBE, and LPS can cause morphological alterations. Shrinkage of the monolayers was shown instead of normal confluent monolayers. Damaged human gingival fibroblasts lost spindle-shaped morphology and changed into round-shaped cells. Cell rounding was also observed in MC3T3-E1 and NIH3T3 cells. Detachment of the cells from each other and the surface of the cultrue dish due to the loss of intercelluar connection caused cellular aggregations. Cells were easily removed from culture dish and tened to float into culture medium without the use of trypsin-EDTA. These morphological findings are the same as other investigations[21,24,25]. MC3T3-E1 exposed to BGS at the dosage of 100㎕ showed rounding deformation of individual cells and displayed granule foramation in cell cytoplasm as well.

There has been variation of expression of cytotoxicity in the choice of cell types. Browne[15] demonstrated that most materials demonstrate greater toxicity when tested with mouse peritoneal macrophages than with BHK C-21 derived fibroblasts. Pissiotis and Spangberg[23] have observed that sonicated extracts of *B. gingivalis* have a different effect on primary cultures of pulp fibroblasts than to established cell line of murine fibroblast L929. Johansson et al[13] showed *P. gingivalis* 33277 were strongly cytotoxic on human gingival fibroblasts in comparison cell lines of epithelial origin. In this study, NIH3T3 cells are specifically sensitive to BGS and SBE compared to other two cell types. There was, however, no difference in the expression of cytotoxicity on three cell types exposed to LPS.

The cytotoxicity casued by sonicated bacterial extracts, LPS and enzymes from *B. gingivalis* was explained by the decrease in growth and proliferation in other investigations[14,25,36]. In this study we observed that each bacterial preparation affected cell viability demonstrating that these preparations could directly affect cells. Moreover, LPS might be one of important factors in the development of pulp/pericapical lesions inducing alveolar bone resorption via production of inflammatory mediators and a variety of cytokines. Future study on the toxicity of *P. nigrescens* including virulence factors and production of bone resorptive cytokines would be helpful in clarifying the role of *P. nigrescens* in infections of endodontic origin.

Ⅴ. CONCLUSION

Gram-negative black-pigmented anaerobic bacteria are the predominant microorganisms associated with acute signs and symptoms of periapical lesions; bacterial components and products are thought to be associated with the pathogenesis of periapical periodontitis. The purpose of this study was to investigate the toxic effets of *P. nigrescens* ATCC type strain and *P. nigrescens* isolated from infected root canals having acute signs and symptoms. Cytotoxic effects of bacterial growth medium supernatants, sonicated bacterial extracts and LPS were analyzed using MTT cell viability test. Two cell lines, MC3T3-E1 and NIH3T3, and human gingival fibroblast were used to compare the possible specific sensitivity of their cytotoxicity against cell types. Morphological changes due to cell damage were observed under inverted microscopy.

The bacterial growth medium supernatants significantly inhibited cell viability of the three cell types at a dosage of 100㎕ ($p<0.05$). Sonicated

bacterial extracts had cytotoxicity on NIH3T3 at both dosages of 12.5㎍/㎖ and 25㎍/㎖ ($p<0.05$). Cytotoxic effects of LPS on three cell types were different according to the concentration of LPS and strain of bacteria. Shrinkage of the monolayers, cellular aggregations and detachment of cells were evident in severely damaged experimental groups under the microscopy.

These results suggest that *P. nigrescens* might be one of causative bacteria contributing to the development and progression of periapical lesions via modulation of host response.

REFERENCES

1. Korzen BH, Krakow AA, Green DB. Pulpal and periapical tissue responses in conventional and monoinfected gnotobiotic rats. Oral Surg Oral Med Oral Pathol 1974;37:783-802.
2. Farber P, Seltzer S. Endodontic microbiology. I. Etiology. J Endodon 1988;14:363-71.
3. Sundqvist G. Taxonomy, ecology, and pathogenicity of the root canal flora. Oral Surg Oral Med Oral Pathol 1994;78:522-30.
4. Sundqvist G. Bacteriological studies of necrotic dental pulps [thesis] Umea, Sweden:Umea university. 1976.
5. Tani-Ishii N, Wang CY, Tanner A, Stashenko P. Changes in root canal microbiota during the development of rat periapical lesions. Oral Microbiol Immunol 1994;9:129-35.
6. van Winkelhoff AJ, Carlee AW, de Graaff J. *Bacteroides endodontalis* and other black-pigmented *Bacteroides* species in odontogenic abscesses. Infect Immun 1985;49:494-8.
7. Stashenko P, Teles R, D'Souza R. Periapical inflammatory responses and their modulation. Crit Rev Oral Biol Med 1998;9:498-521.
8. Stashenko P. Regulatory effect of monocytes on T cell proliferative responses to oral microbial antigens. Infect Immun 1982;38:938-47.
9. Torabinejad M, Clagett J, Engel D. A cat model for evaluation of mechanisms of bone resorption: induction of bone loss by simulated immune complexes and inhibition by indomethacin. Calcif Tissue Int 1979;29:207-14.
10. Shah HN, Collins DM. *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. Int J Syst Bacteriol 1990;40:205-8.
11. Gharbia SE, Haapasalo M, Shah HN, Kotiranta A, Lounatmaa K, Pearce MA, Devine DA. Characterization of *Prevotella intermedia* and *Prevotella nigrescens* isolates from periodontic and endodontic infections. J Periodontol 1994;65:56-61.
12. Baumgartner JC, Watkins BJ, Bae KS, Xia T. Association of black-pigmented bacteria with endodontic infections. J Endodon 1999;25:413-5.
13. Johansson A, Bergenholtz A, Holm SE. Strong cytotoxicity to human gingival fibroblasts by *Porphyromonas gingivalis* ATCC 33277. J Periodont Res 1996;31:477-82.
14. Yamasaki M, Nakata K, Imaisumi I, Iwama A, Nakane A, Nakamura H. Cytotoxic effect of endodontic bacteria on periapical fibroblasts. J Endodon 1998;24:534-9.
15. Browne RM. The *in vitro* assessment of the cytotoxicity of dental materials- does it have a role? Int Endod J 1988;21:50-8.
16. Baumgartner JC, Watkins BJ, Bae K-S, Xia T. Assoication of black-pigmented bacteria with endodontic infections. J Endodon 1999;25:413-5.
17. Eidhin DN, Mouton C. A rapid method for preparation of rough and smooth lipopolysaccjaride from Bacteroides, Porphyromonas and Prevotella. FEMS Microbiol Lett 1993;110:133-8.
18. Sundqvist G, Johansson E, Sjogren U. Prevalence of black-pigmented bacteria with endodontic infections. J Endodon 1989;15:13-9.
19. Frandsen EVG, Poulsen K, Kilian M. Confirmation of the species *Prevotella intermedia* and *Prevotella nigrescens*. Int J Sys Bact 1995;45:429-35.
20. Bae KS, Baumgartner JC, Shearer TR, David LL. Occurrence of *Prevotella nigrescens* and *Prevotella intermedia* in infections of endodontic origin. J Endod 1997;23:620-3.
21. van Steenbergen T, Ouden D, Touw JA, Graarr JD. Cytotoxic activity of *Bacteroides gingivalis* and *Bacteroides asaccharolyticus*. J Med Microbiol 1982;5:253-8.
22. Shah HN, Gharbia SE, O'Toole CM. Assessment of the relative cytotoxicity of *Porphyromonas gingivalis* cells, products, and components on human epithelial cell lines. J Periodontol 1992;63:44-51.
23. Larjava H, Uitto VJ. Effects of extracts from *Bacteroides gingivalis*, *Bacteroides intermedius*, and *Bacteroides asaccharolyticus* on the growth of fibroblast lines obtained from healthy and inflamed human gingiva. Oral Microbiol Immunol 1987;2:112-6.
24. Stevens RH, Hammond BF. The comparative cytotoxicity of periodontal bacteria. J Periodontol 1988;59:741-9.
25. Pissiotis E, Spangberg LSW. Toxicity of sonicated extracts of *Bacteroides gingivalis* on human pulpal cells and L929 cells in vitro. J Endodon 1991;17:553-60.
26. Hausmann E, Weinfeld N, Miller WA. Effects of lipopolysaccharides on bone resorption in tissue culture. Cal Tissue Res 1972;9:272-82.
27. Schein B, Shilder H. Endotoxin content in endodontically involved teeth. J Endodon 1975;1:19-21.
28. Horiba N, Maekawa Y, Yamauchi Y, Ito M, Matsumoto T, Nakamura H. Complement activation by lipopolysaccharides purified from gram-negative bacteria isolated from infected root canals. Oral Surg Oral Med Oral Pathol 1992;74:48-51.
29. Coutingho A, Moller G. Thymus-independent B-cell induction and paralysis. Adv Immunol 1975;21:113-36.
30. Burchett SK, Weaver WM, Westall JA, Larsen A, Kronheim S, Wilson CB. Regulation of tumor necrosis factor/cachecin and IL-1 secretion in human mononuclear phagocytes. J Immunol 1988;140:3473-81.
31. Melchers F, Braun V, Galanos C. The lipoprotein of the outer membrane of Escherichia coli: Ab-lymphocyte mitogen. J Exp Med 1975;142:473-82.
32. Dewhirst FE. N-acetyl muramyl dipeptide stimulation of bone resorption in tissue culture. Infect Immun 1982;35:133-7.

33. Morrison DC, Betz SJ, Jacobs DM. Isolation of a lipid A bound polypeptide responsible for "LPS-initiated" mitogenesis of C3H/HeJ spleen cells. J Exp Med 1976;144:840-6.
34. Leigh W, Rahemtulla F, Michalek SM. Interleukin (IL)-1 and *Porphyromonas gingivalis* lipopolysaccharide stimulation of IL-6 production by fibroblasts derived from healthy or periodontally diseased human gingival tissue. J Periodontol 1999;70:274-82.
35. Ebersole JL, Steffen MJ. Human antibody responses to outer enveolpe antigens of *Porphyromonas gingivalis* serotypes. J Periodont Res 1995;30:1-14.
36. Guigand M, Pellen P, Mouton C, Bonnaure-Mallet M. Cytotoxic effect of vesicles produced by *Porphyromonas gingivalis* on fibroblasts in culture. J Periodont Res 1995;30:141-3.

사진부도 ①

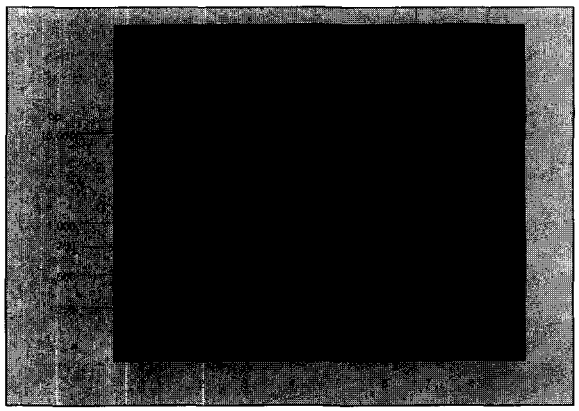

Fig. 1. Agarose gel electrophoresis of PCR products with use of primers from the 16S rRNA gene of *P. nigresences*. Lane 1 shows 1kb DNA ladder. Lane 2 shows *P. nigrescens* ATCC 33563. Lanes 3 to 8 show clinical isolates of *P. nigrescens*; lane 3, 203; lane 4, 304; lane 5, 305; lane 6, 2202; lane 7, 2203;and lane 8, 2302.

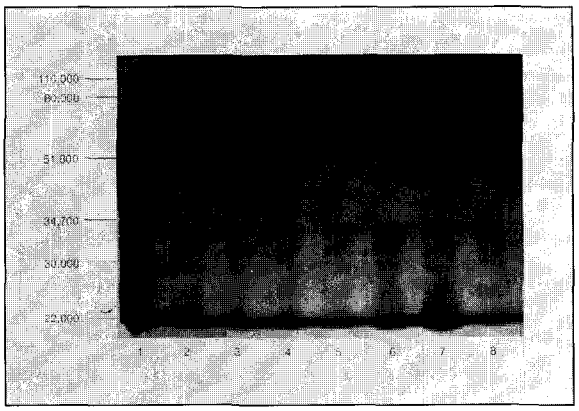

Fig. 2. Silver-stained SDS-PAGE of LPS prepared from *P. nigrescens*. Lane 1 is size marker. Lane 2 shows *P. nigrescens* ATCC 33563. Lanes 3 to 8 show clinical isolates of *P.nigrescens*; lane 3, 203; lane 4, 304; lane 5, 305; lane 6, 2202; lane 7, 2203;and lane 8, 2302.

Fig. 3. MC3T3-E1 osteoblasts in control culture show normal morphogy (×400).

Fig. 4. MC3T3-E1 osteoblasts exposed to 100㎕ of bacterial growth supernatants from *P. nigrescens* ATCC type strain show deformation of cell morphology and display granulation of cells (×400).

사진부도 ②

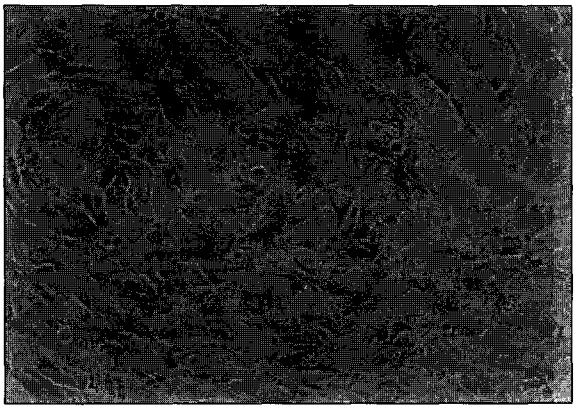

Fig. 5. NIH3T3 fibroblasts in control culture show normal morpholgy (×400).

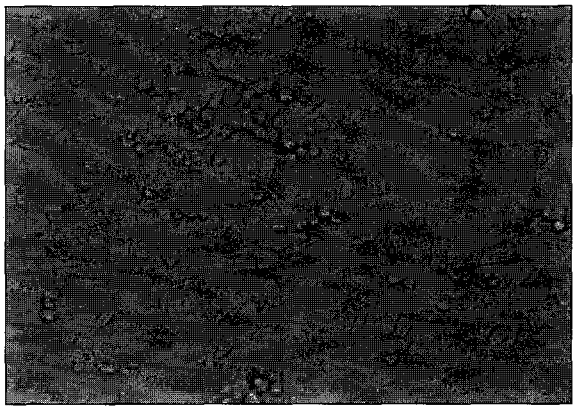

Fig. 6. NIH3T3 fibroblasts exposed to 100㎕ of bacterial growth supernatants from *P. nigrescens* ATCC type strain became rounded and lost intercellular connection (×400).

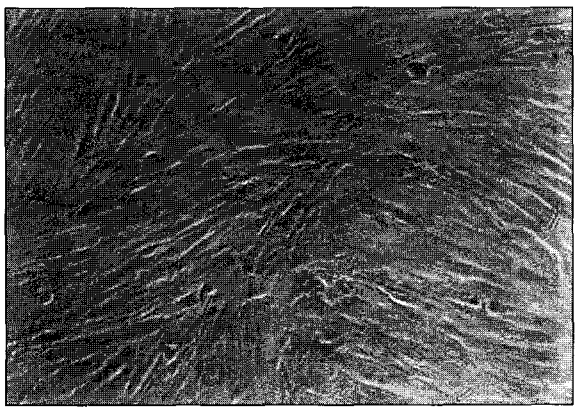

Fig. 7. Human gingival fibroblasts in control culture show typical spindle-shaped morphology (×400).

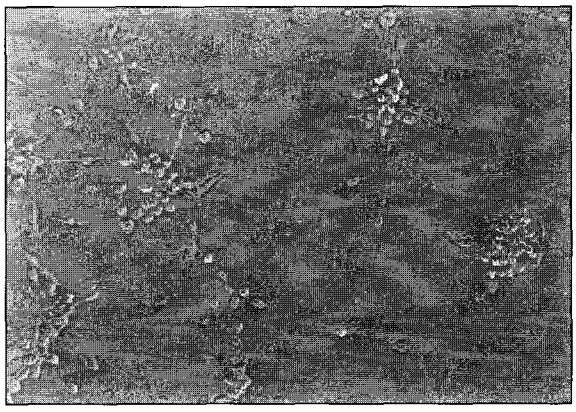

Fig. 8. Human gingival fibroblasts exposed to 2.5 ㎎/㎖ of LPS from clinical isolate 2302 show severe morphological alteration and cell aggregations (×400).