The Inhibitory Effect of Endotoxins on Growth of Human Cell Lines

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Abstract

A study was conducted to examine the effect of endotoxin present in periodontal pockets on the proliferation and attachment of human cell lines on the culture plates (Ca9-22 and gingival fibroblasts).

The endotoxin was collected from periodontal pockets of anterior teeth in patients with periodontal disease by subgingival irrigation with sterilized distilled water. The solutions obtained were then subjected to hot phenol-water extraction. The collected endotoxin from periodontal pocket and four other kinds of endotoxin obtained commercially as positive controls were added to cell cultures and the numbers of viable cells on the culture plates were counted.

Among the commercially available endotoxins used in this study, only 500 $\mu\text{g/ml}$ of endotoxin derived from *Escherichia coli* 0111:B4 significantly decreased the number of attachment cells of Ca9-22 and gingival fibroblasts on the culture plates.

Endotoxin from periodontal pockets at 5 $\mu\text{g/ml}$ also significantly decreased the numbers of attachment cells of both cell lines.

Introduction

The pathological potential of endotoxin derived from Gram-negative bacteria is now well known^[1-3]. Endotoxin has been implicated in the initiation and progression of periodontal tissue destruction^[4-7].

Yoshinuma^[8] reported that the solution collected from periodontal pockets by subgingival irrigation contained endotoxin, and that such irrigation decreased the amount of endotoxin in periodontal pockets and improved gingival inflammation.

The purpose of this study was to evaluate the cytotoxicity of endotoxin extracted from periodontal pocket irrigant on two human cell lines (Ca9-22 and gingival fibroblasts).

Materials and Methods

1. Materials

1) Subjects

The subjects of this study were patients with moderate to severe periodontal disease, who had been referred to Nihon University School of Dentistry.

After a comprehensive periodontal examination, patients showing ≥ 4 mm attachment loss at maxillary anterior teeth were selected. Patients with systemic disorders or a history of antibiotic use in the previous 6 months were excluded.

Finally, ten patients, five males and five females, aged 29 to 56 years, were recruited.

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Fig.1 Experimental design

2) Cell lines

Ca9-22 cells, originally derived from human oral carcinoma, were supplied by Tokyo Medical and Dental University. The human gingival fibroblasts employed were derived from an adult patient with a healthy marginal gingiva.

The cells were maintained in α -MEM supplemented with 10% fetal bovine serum and incubated at 37°C in 5%CO₂ in an incubator.

3) Commercially available endotoxins

Endotoxin derived from *Escherichia coli* 0111:B4, *Escherichia coli* 055:5B, *Salmonella typhimurium* and *Salmonella enteritidis* (Difco Labs., U.S.A.) were used.

2. Methods

1) Subgingival irrigation

One week after periodontal examination, subgingival irrigation of the periodontal pockets was performed by the method of Hardy et al.^[9]

Periodontal pockets of anterior teeth were irrigated with sterilized distilled water using a 10 ml disposable syringe fitted with a 1-inch conical Luer stainless steel 23 G hypodermic needle. The needle was shortened to 2.2 cm by removing the bevelled end and then bent along its shank to an angle of approximately 130°.

The needle was inserted into the periodontal pocket 3 mm from the gingival margin. With firm hand pressure, each pocket was then irrigated with 10 ml of distilled water. The irrigation procedure was repeated three times for each tooth.

The irrigants were collected using a funnel held near the maxillary incisor during irrigation. The irrigants from different subjects were stored at -20°C until the next procedure.

2) Extraction of endotoxin

All the samples from the different subjects were pooled and then lyophilized, and the endotoxin was extracted according to the hot phenol-water procedure of Westphal et al.^[10]

3) Detection of endotoxin

For the Lymulus lysate assay, Pyrodick (Teikoku Zouki, Tokyo, Japan) was used to determine the biological activity of endotoxin in the sample.

After the detection of endotoxin, it was dried and restored at -20°C.

4) Inhibitory effect of commercially available endotoxins on cell growth

As a positive control experiment, the inhibitory effect of commercially available endotoxins on human cell growth was investigated.

The experimental design is shown in Fig. 1. Ca9-22 and gingival fibroblasts suspended in α -MEM were inoculated in 96-well tissue culture plates (5×10^3 cells/well). After 24 h, the commercially available endotoxins were added to the wells at the concentrations of 5, 50 and 500 $\mu\text{g/ml}$ using α -MEM. After 96 h, the numbers of cells were counted using a microcellcounter.

5) Inhibitory effect of periodontal endotoxin on cell growth

The experimental design was the same as that used for the commercially available endotoxins. Ca9-22 and gingival fibroblasts were prepared in tissue culture plates (5×10^3 cells/well).

After 24 h from inoculation of human cell lines, the concentrations of periodontal endotoxin and endotoxin from *E.coli* 0111:B4 were adjusted to 5, 5×10^{-1} and 5×10^{-2} $\mu\text{g/ml}$ in culture medium.

3. Statistical analysis

All statistical evaluations were performed by Student's *t* test.

Results

1) Inhibitory effect of commercially available endotoxins on cell growth

Representative results of this study are shown in Tables 1 and 2.

The number of Ca9-22 cell was $(10.1 \pm 0.8) \times 10^4$ cells/well in the negative control, and that at a concentration of 500 $\mu\text{g/ml}$ of endotoxin from *E.coli* 0111:B4 was $(6.4 \pm 1.3) \times 10^4$ cells/well.

The number of Ca9-22 cells was decreased significantly in comparison with the control at a concentration of 500 $\mu\text{g/ml}$ of endotoxin from *E.coli* 0111B:4.

The number of gingival fibroblasts was $(21.8 \pm 2.3) \times 10^4$ cells/well in the negative control, and that at a concentration of 500 $\mu\text{g/ml}$ of endotoxin from *E.coli* 0111:B4 was $(14.4 \pm 3.8) \times 10^4$ cells/well. The number of gingival fibroblasts was decreased significantly in comparison with the control at a concentration of 500 $\mu\text{g/ml}$ of endotoxin from *E.coli* 0111:B4.

2) Inhibitory effect of periodontal endotoxin on cell growth

Representative results of this experiment are shown in Tables 3 and 4. The number of Ca9-22 cells was $(8.8 \pm 1.5) \times 10^4$ cells/well in the negative control, whereas that at a concentration of 5 $\mu\text{g/ml}$ of periodontal endotoxin was $(5.5 \pm 0.6) \times 10^4$ cells/well.

The number of Ca9-22 cells was decreased significantly in comparison with the control at a concentration of 5 $\mu\text{g/ml}$ of periodontal endotoxin.

The number of gingival fibroblasts was $(18.2 \pm 0.8) \times 10^4$ cells/well in the negative control, and that at a concentration of 5 $\mu\text{g/ml}$ of periodontal endotoxin was $(10.1 \pm 1.1) \times 10^4$ cells/well.

The number of gingival fibroblasts was decreased significantly in comparison with the control at a concentration of 5 $\mu\text{g/ml}$ periodontal endotoxin.

Table 1 Inhibitory effects of the commercially available endotoxins on cell growth of Ca9-22

	500 $\mu\text{g/ml}$	50 $\mu\text{g/ml}$	5 $\mu\text{g/ml}$	control
<i>E.coli</i> 0111:B4	6.4 ± 1.3	10.1 ± 1.9	10.2 ± 2.2	10.1 ± 0.8
<i>E.coli</i> 055:5B	12.4 ± 1.1	12.3 ± 0.4	11.3 ± 1.2	
<i>S.typhimurium</i>	12.6 ± 0.9	13.9 ± 3.6	13.7 ± 1.8	
<i>S.enteritidis</i>	11.3 ± 2.8	11.1 ± 0.3	11.0 ± 0.9	

(Mean $\pm$ SD) $\times 10^4$ cells / well
n=5

○:P < 0.01

Table 2 Inhibitory effects of the commercially available endotoxins on cell growth of gingival fibroblasts

	500 $\mu\text{g/ml}$	50 $\mu\text{g/ml}$	5 $\mu\text{g/ml}$	control
<i>E.coli</i> 0111:B4	14.4 $\pm$ 3.8	24.0 $\pm$ 3.8	24.4 $\pm$ 4.8	21.8 $\pm$ 2.3
<i>E.coli</i> 055:5B	22.8 $\pm$ 4.0	24.4 $\pm$ 3.8	24.6 $\pm$ 3.4	
<i>S.typhimurium</i>	25.0 $\pm$ 1.6	25.0 $\pm$ 2.3	22.4 $\pm$ 1.8	
<i>S.enteritidis</i>	23.0 $\pm$ 3.6	22.8 $\pm$ 3.5	23.2 $\pm$ 3.3	

(Mean $\pm$ SD) $\times 10^4$ cells / well
n=5
○: P < 0.01

Table 3 Inhibitory effect of periodontal endotoxin on cell growth of Ca9-22

	5 $\mu\text{g/ml}$	5 $\times 10^{-1}$ $\mu\text{g/ml}$	5 $\times 10^{-2}$ $\mu\text{g/ml}$	control
<i>E.coli</i> 0111:B4	9.1 $\pm$ 1.2	8.1 $\pm$ 0.5	7.9 $\pm$ 0.3	
P.E.	5.5 $\pm$ 0.6	7.0 $\pm$ 0.4	7.9 $\pm$ 1.0	8.8 $\pm$ 1.5

(Mean $\pm$ SD) $\times 10^4$ cells / well
n=5
○: P < 0.01
P.E. (Periodontal endotoxin)

Table 4 Inhibitory effect of periodontal endotoxin on cell growth of gingival fibroblasts

	5 $\mu\text{g/ml}$	5 $\times 10^{-1}$ $\mu\text{g/ml}$	5 $\times 10^{-2}$ $\mu\text{g/ml}$	control
<i>E.coli</i> 0111:B4	19.0 $\pm$ 1.6	18.6 $\pm$ 0.6	17.4 $\pm$ 0.5	
P.E.	10.4 $\pm$ 1.1	16.0 $\pm$ 1.4	17.6 $\pm$ 3.3	18.2 $\pm$ 0.8

(Mean $\pm$ SD) $\times 10^4$ cells / well
n=5
○: P < 0.01
P.E. (Periodontal endotoxin)

Discussion

It is well known that Gram-negative bacteria are dominant in the subgingival microflora of deep periodontal pocket^[11-14]. Fine et al.^[15] reported that the endotoxin of loosely adherent plaque^[15] was more biologically active than that of firmly adherent plaque. Moore et al.^[16] demonstrated that 99% of endotoxin of root surface-associated material on periodontally diseased teeth could be removed by a comparatively gentle procedure. However, few studies have suggested that the endotoxin from periodontal pathogenic bacteria might show cytotoxicity against oral cell lines.

Therefore, we extracted endotoxin from irrigant solution obtained by subgingival irrigation and evaluated its inhibitory effect on human cell growth. Singer et al.^[17] reported that inhibition of L-929 cell

proliferation by endotoxin from *E.coli* 0127:B13 occurred at a concentration of 100 $\mu\text{g/ml}$. Frank et al.^[18] investigated alterations in mitochondria of human gingival fibroblasts upon exposure to medium containing 500 $\mu\text{g/ml}$ of endotoxin from *E.coli*. Accordingly, we investigated the effects of commercially available endotoxins as a positive control, and found that 500 $\mu\text{g/ml}$ of endotoxin from *E.coli* 0111:B4 showed the most inhibitory effect on cell growth. Therefore, we employed this endotoxin as a positive control.

Our study demonstrated that the cytotoxicity of endotoxin from periodontal pockets was greater than that of endotoxin from *E.coli* 0111:B4, and that the number of Ca9-22 and gingival fibroblasts decreased significantly in the presence of concentration of 5 $\mu\text{g/ml}$ endotoxin from periodontal pockets in comparison with the negative control.

Horiba et al.^[19] reported that the cytotoxicity of endotoxin from *Fusobacterium nucleatum* is greater than that of endotoxin from *E.coli* 0111:B4. Therefore, it is thought that the cytotoxicity of endotoxin from periodontal pockets, as it includes endotoxin of periodontal pathologic bacteria such as *F.nucleatum*, is greater than that of endotoxin from *E.coli* 0111:B4. Sugizaki^[20] reported that endotoxins prepared from *Porphyromonas gingivalis* (*Bacteroides gingivalis*) and *Prevotella intermedium* (*Bacteroides intermedium*) at a concentration of 10 $\mu\text{g/ml}$ altered the activity of glucose-6-phosphate dehydrogenase in Gin-1 cell.

Tagata^[21] found that the endotoxins from *P. intermedium* and *P. gingivalis* accelerated collagenase activity. Thus it is possible that the endotoxin from periodontal pockets may inhibit cell growth by altering cellular metabolism.

Conclusion

We extracted endotoxin from periodontal pockets by subgingival irrigation, and evaluated its inhibitory effect on cell growth. It was found that:

1. Among commercially available endotoxins, the numbers of Ca9-22 and gingival fibroblasts were decreased significantly at a concentration of 500 $\mu\text{g/ml}$ of endotoxin from *E.coli* 0111:B4 in comparison with the control.
 2. The numbers of Ca9-22 and gingival fibroblasts were decreased significantly at a concentration of 5 $\mu\text{g/ml}$ of periodontal endotoxin in comparison with the control.
- Therefore, the cytotoxicity of endotoxin from periodontal pockets is greater than that of endotoxin from *E.coli* 0111:B4.

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