

## *Porphyromonas gingivalis* lipopolysaccharide activated bone resorption of osteoclasts by inducing IL-1, TNF, and PGE<sup>1</sup>

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**KEY WORDS** bone resorption; interleukin-1; tumor necrosis factor; prostaglandins E; osteoclasts; *Porphyromonas gingivalis*; lipopolysaccharides; periodontitis

### ABSTRACT

**AIM:** To study the effects of *Porphyromonas gingivalis* lipopolysaccharide (Pg-LPS) on inducing interleukin-1 (IL-1), tumor necrosis factor (TNF), prostaglandin E (PGE), and activating osteoclasts, in order to understand mechanism of osteoclast activation. **METHODS:** Pg-LPS was prepared by phenol-water method. IL-1, TNF, and PGE induced by Pg-LPS were isolated by chromatography. Ca<sup>2+</sup> concentration was detected by atomic absorption spectrophotometry. Acid phosphatase and carbonic anhydrase in periodontal membranes were examined by histochemistry. **RESULTS:** Pg-LPS was able to stimulate peripheral blood mononuclear cells (PBMC) or the cells from human periodontal tissue secreting IL-1, TNF, and PGE. The outputs of these cytokines were increased in pace with the enhancement of Pg-LPS at the dose range of 0.5-5.0 mg/L. All of the three cytokines showed activities of accelerating Ca<sup>2+</sup> release from rat calvarial bones, and the activity of PGE was the strongest. The amounts of both the acid phosphatase and carbonic anhydrase in the periodontal membranes of Pg-LPS injected rats were obviously increased ( $P < 0.01$ ). In the periodontal membranes of Pg-LPS injected rats, the amount of activated osteoclasts were obviously increased in pace with Pg-LPS injection times ( $P < 0.01$ ). However, the activating rates of osteoclasts were stable to approximately 65 % because of the increase of inactivated osteoclasts. **CONCLUSION:**

Pg-LPS possessed strong activities to induce human PBMC and the cells from human periodontal tissue to produce IL-1, TNF, and PGE in a dose-dependent manner within a certain concentration range of the LPS. Pg-LPS could efficiently activate osteoclasts, and the mechanism of osteoclast activation was probably associated with the increase of acid phosphatase and carbonic anhydrase.

### INTRODUCTION

Periodontitis is a major cause of anodontia for adults. Alveolar bone resorption is the most important clinical sign and pathologic change of the disease. Enhancement of the amount and activity of osteoclasts in periodontal membrane was considered to be responsible for alveolar bone resorption<sup>1-31</sup>. *Porphyromonas gingivalis* (*P. gingivalis*), the most important causative agent of chronic periodontitis<sup>1-63</sup>, possesses lipopolysaccharide with endotoxin activity<sup>7,81</sup>. Interleukin-1 (IL-1), tumor necrosis factor (TNF), and prostaglandin E (PGE) induced by *Escherichia coli* (*E. coli*) lipopolysaccharide were demonstrated to mediate bone resorption<sup>9,101</sup>. However, *E. coli* was not a dominant bacterium in patients' periodontal pockets<sup>5,101</sup>. Recent literatures reported that *P. gingivalis* lipopolysaccharide had the ability to induce IL-1 and TNF<sup>11,121</sup>. In spite of these advances, the relation between the concentration of *P. gingivalis* lipopolysaccharide and the effects on inducing IL-1, TNF and PGE, the potentials of the three cytokines on stimulating bone resorption, and the mechanisms of lipopolysaccharides activating osteoclasts were little understood.

In the present study, the dose-effect relationship of *P. gingivalis* lipopolysaccharide on inducing IL-1, TNF, and PGE, as well as activating osteoclasts, and the abilities of the three cytokines on mediating bone resorption were investigated. Furthermore, the mechanism of osteoclast activation using *P. gingivalis* lipopolysaccharide was also studied.

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## MATERIALS AND METHODS

**LPS preparation** BHI medium (Oxoid, USA) containing 5 % sheep blood was used to culture *P. gingivalis*. Lipopolysaccharide of *P. gingivalis* strain ATCC 33277 (Pg-LPS) was extracted by phenol-water method<sup>[13]</sup> and then purified by RNase digestion, incubated at 100 °C for 10 min and ultracentrifuged at 110 000 × g for 3 h.

**Peripheral blood mononuclear cell (PBMC) isolation** The PBMC of normal adults were isolated by HISTOPAQUE-1077 (Sigma) with a density of 1.007 kg/L, and suspended in RPMI 1640 medium (Gibco) containing 10 % bovine serum (Hyclone) to the final cell concentration of  $1 \times 10^6$ /L.

**Isolation of the cells from human gingival tissue** Human gingival tissue isolated from patients' impacted molars were digested with 0.5 % trypsin-0.02 % edetic acid solution at 37 °C for 30 min. The cells from the gingival tissue were cultured in RPMI 1640 medium containing 10 % bovine serum under 5 % CO<sub>2</sub> atmosphere to form cell monolayers. The cell monolayers were digested with 0.05 % trypsin-0.02 % edetic acid solution, and then suspended in RPMI 1640 medium containing 10 % bovine serum to the final cell concentration of  $1 \times 10^7$ /L.

**Animals** The SD rats used in this study were offered by the Center of Experimental Animals, Medical School of Zhejiang University (Grade II, Certificate No 22-9601018). The newborn rats (10.0 g ± 1.0 g) were used for bone resorption test and the rats (100 g ± 10 g) were used for examinations of acid phosphatase, carbonic anhydrase, and osteoclasts. All of the rats were randomly divided into groups.

**Preparation of IL-1, TNF, and PGE** Pg-LPS 0.5, 1.0, 5.0, and 10 mg/L were added respectively into the wells of cell culture plates containing the suspension of PBMC or the suspension of gingival tissue cells. These plates were incubated under 5 % CO<sub>2</sub> atmosphere at 37 °C for 12, 16, and 48 h, respectively.

The supernatant of the PBMC culture after 48 h incubation went through Sephadex G-75 column for IL-1 isolation. The elution solution was RPMI 1640 medium without bovine serum. The activities of the eluted protein fragments stimulating the proliferation of thymocytes from SD rats were detected by <sup>3</sup>H-TdR incorporation. The fragment with obvious proliferating activity was considered as partly purified IL-1<sup>[14]</sup>.

The supernatant of the PBMC culture after 16 h in-

incubation went through DEAE-52 column for TNF isolation. The elution solution was 0.01 mol/L PBS (pH 8.0). The cytotoxicities of the eluted protein fragments were detected by using toxicity test on mouse L929 cells. The fragment with obvious cytotoxicity activity was considered as partly purified TNF<sup>[15]</sup>.

The cell culture from human gingival tissue after 12 h incubation was used for PGE extraction according to Sweet's method<sup>[16]</sup>.

**Bone resorption test** Each of the calvarial bones from newborn SD rats was cultured in a well of cell culture plates containing 2 mL of RPMI 1640 medium under 5 % CO<sub>2</sub> atmosphere at 37 °C for 24 h. The medium in the wells was discarded after incubation. RPMI 1640 medium 2 mL containing 10 mg/L of IL-1, TNF, PGE, or Pg-LPS preparations was added respectively into the wells. The same volume of the medium without the cytokines and Pg-LPS was also added into the wells as a negative control. All of the plates were incubated under 5 % CO<sub>2</sub> atmosphere at 37 °C for 48 h. Each of the tested groups mentioned above contained 8 wells.

For measurement of bone resorption effects caused by the three cytokines and Pg-LPS, the Ca<sup>2+</sup> concentrations in the medium of the wells were detected by atomic absorption spectrophotometry after the last incubation.

**Amount and activity of osteoclasts in rat periodontal membranes** The SD rats in four tested groups were injected with 0.1 mL of Pg-LPS solution (1 mg/kg) into the labial surface of alveolar bone between the two inferior anterior teeth for 1, 2, 3, or 1 times respectively. Another group of SD rats was injected with the same volume of normal saline for 4 times as a negative control. Each of the five groups contained 8 animals. The interval between two injections was 5 d.

The mandible bones containing teeth from the rats were fixed with formaldehyde-calcium solution and then were demineralized on the 5th day after the final injection. The amount of osteoclasts in periodontal membranes in the mandible bone sections after HE staining were examined under microscope (× 400). An osteoclast was considered to be active if its nucleus numbers were more than three and its cytoplasm showed strong eosinophilia. Activating rate of the osteoclasts was calculated by the following formula: Activating rate = (Activated osteoclast number/Total osteoclast number) × 100 %.

**Examinations of acid phosphatase and carbonic anhydrase** The levels of acid phosphatase and

carbonic anhydrase in periodontal membranes of the mandible bone sections mentioned above were examined by using Gomori's method and Hansson's methods respectively<sup>[17]</sup>. The particles of acid phosphatase and carbonic anhydrase with black color could be seen under microscope ( $\times 400$ ). For each of the specimens, acid phosphatase particles or carbonic anhydrase particles in ten fields of view were counted.

**Data analysis** The results of bone resorption test and the examinations of acid phosphatase and carbonic anhydrase were reported as  $\bar{x} \pm s$  and all values were compared by *t*-test. The activating rates of osteoclasts were analyzed by  $\chi^2$ -test.

## RESULTS

**Ability of Pg-LPS on IL-1, TNF, and PGE induction** Although all of the four doses of Pg-LPS used in this study could induce IL-1, TNF, and PGE, 5 mg/L of Pg-LPS seem to be the optimal concentration to induce these cytokines (Tab 1).

**Tab 1.** Activities of Pg-LPS on inducing IL-1, TNF, and PGE in PBMC by chromatography.  $n = 3$  experiments.  $\bar{x} \pm s$ .

| Pg-LPS concentration/ $\text{mg}\cdot\text{L}^{-1}$ | Output/ $\text{mg}\cdot\text{L}^{-1}$ |               |                 |
|---|---------------------------------------|---------------|-----------------|
|   | IL-1 (48 h)                           | TNF (16 h)    | PGE (12 h)      |
| 0.5   | $5.3 \pm 0.9$                         | $3.4 \pm 0.7$ | $0.06 \pm 0.03$ |
| 1.0   | $6.7 \pm 0.5$                         | $4.2 \pm 0.6$ | $0.22 \pm 0.06$ |
| 5.0   | $9.0 \pm 1.2$                         | $4.9 \pm 0.7$ | $1.52 \pm 0.35$ |
| 10.0  | $6.3 \pm 0.7$                         | $3.5 \pm 0.6$ | $0.30 \pm 0.07$ |

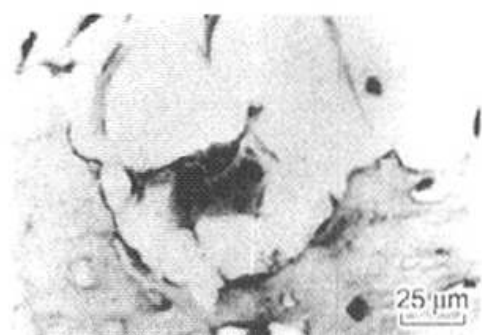
**Effect of IL-1, TNF, PGE and Pg-LPS on bone resorption stimulation** The abilities of IL-1, TNF, and Pg-LPS to stimulate  $\text{Ca}^{2+}$  release were similar to each other ( $P > 0.05$ ). PGE seems to possess the strongest ability to induce  $\text{Ca}^{2+}$  release in comparison with the other two cytokines and Pg-LPS ( $P < 0.05$ ). However, IL-1, TNF, PGE, and Pg-LPS could obviously stimulate the  $\text{Ca}^{2+}$  release from rat calvarial bones compared with control ( $P < 0.01$ , Tab 2).

### Counting of total and activated osteoclasts

Osteoclasts in periodontal membranes of SD rats injected with Pg-LPS were obviously activated (Fig 1). The activating rates of osteoclasts in the animal groups injected with Pg-LPS were marked higher than that of control group. Although the activated osteoclast numbers in the Pg-LPS injected groups were increased in pace with

**Tab 2.**  $\text{Ca}^{2+}$  release of rat calvarial bones caused by IL-1, TNF, PGE, and Pg-LPS.  $n = 8$  rats.  $\bar{x} \pm s$ .  $^*P < 0.05$  vs PGE.  $^{**}P < 0.01$  vs control.

| Group   | Range of $\text{Ca}^{2+}$ concentration/ $\text{mg}\cdot\text{L}^{-1}$ | $\text{Ca}^{2+}$ concentration/ $\text{mg}\cdot\text{L}^{-1}$ |
|---------|--|---|
| Control | 16.4-21.3  | $18.5 \pm 2.9$  |
| IL-1    | 19.8-23.9  | $21.9 \pm 2.1^{**}$   |
| TNF     | 20.1-27.9  | $23.2 \pm 3.0^{**}$   |
| PGE     | 24.4-29.2  | $26.4 \pm 2.4^*$  |
| Pg-LPS  | 21.5-23.6  | $22.4 \pm 1.2^{**}$   |



**Fig 1.** Activated osteoclast in rat periodontal membrane after Pg-LPS injection (1 mg/kg  $\times$  4 times) at labial surface of alveolar bone.  $\times 400$ .

Pg-LPS injection times, the activating rates were stable to approximately 65% because of the increase of inactivated osteoclast numbers (Tab 3).

**Tab 3.** Total numbers and activating rates of rat osteoclasts after Pg-LPS injection.

| Group        | Injection times | Total activated osteoclasts/total inactivated osteoclasts | Activating rate/% |
|--------------|-----------------|---|-------------------|
| Control (NS) | 4               | 8/21  | 27.6              |
| Pg-LPS       | 1               | 32/22   | 59.3              |
|              | 2               | 55/28   | 66.3              |
|              | 3               | 75/37   | 66.9              |
|              | 4               | 173/67  | 61.7              |

**Changes of acid phosphatase and carbonic anhydrase** The numbers of acid phosphatase particles and carbonic anhydrase particles in the rat periodontal membranes of Pg-LPS injected groups were significantly higher than that of control group ( $P < 0.01$ ). However, the acid phosphatase particles were obviously increased in pace with Pg-LPS injection times ( $P < 0.01$ )

but the carbonic anhydrase particles were not (Tab 4).

**Tab 4. Changes of acid phosphatase and carbonic anhydrase particles in periodont membranes of Pg-LPS in injected rats.  $n=8$ .  $\bar{x} \pm s$ .  $^*P < 0.01$  vs control.**

| Groups       | Injection times | Mean of enzyme particles |                         |
|--------------|-----------------|--------------------------|-------------------------|
|              |                 | Acid phosphatase         | Carbonic anhydrase      |
| Pg-LPS       | 1               | 11.4 ± 1.7 <sup>c</sup>  | 7.4 ± 2.1 <sup>c</sup>  |
|              | 2               | 17.9 ± 1.9 <sup>e</sup>  | 9.5 ± 1.9 <sup>e</sup>  |
|              | 3               | 23.1 ± 2.0 <sup>e</sup>  | 8.5 ± 2.2 <sup>e</sup>  |
|              | 4               | 27.5 ± 2.4 <sup>e</sup>  | 10.4 ± 2.0 <sup>e</sup> |
| Control (NS) | 4               | 5.0 ± 1.3                | 1.4 ± 0.9               |

## DISCUSSION

In the present study, Pg-LPS at low concentrations (0.5 - 10.0 mg/L) could efficiently induce human PBMC and the cells from human gingival tissue to produce IL-1, TNF, and PGE respectively. The inducing effects were dose-dependent within 0.5 - 5.0 mg/L of Pg-LPS. However, Pg-LPS 10.0 mg/L compared to the dose of 5.0 mg/L resulted in the output fall of all the three cytokines. This phenomenon suggested that the Pg-LPS at a high dose suppressed these cells to synthesize the cytokines probably due to the cytotoxicity<sup>[18]</sup>. Since lipopolysaccharide concentration in periodontal pockets and in dental alveoli of periodontitis patients were not very high<sup>[8]</sup>, the effect of Pg-LPS at low doses on cytokine inducement found in this study is clinically significant.

The result of bone resorption test in this study revealed that IL-1, TNF, and PGE induced by Pg-LPS could efficiently stimulate the rat calvarial bones to release  $Ca^{2+}$ . PGE possessed the strongest stimulating effect compared with the other two cytokines ( $P < 0.01$ ). The direct effect of Pg-LPS on inducing  $Ca^{2+}$  release suggested that Pg-LPS might induce cytokines rapidly or damage bone tissue directly.

The activating rates as well as the total numbers of osteoclasts in the periodontal membranes from Pg-LPS injected rats were significantly increased compared with control group ( $P < 0.01$ ). Although the activated osteoclast numbers were continuously increased in pace with Pg-LPS injection times, the osteoclast activating rates were stable to approximate 65 % because of the increase of inactivated osteoclast numbers. This result indicated that Pg-LPS possessed not only the effect to activating os-

teoclasts but also showed a potential to stimulate the differentiation of pro-osteoclasts into osteoclasts.

Acid phosphatase and carbonic anhydrase numerously exist in osteoclasts but are rare in the other cells of periodontal membrane tissue<sup>[17]</sup>. Therefore, assays for the two enzymes can be used to identify osteoclasts and to measure the cell's activity. In the present study, the numbers of acid phosphatase and carbonic anhydrase particles in the periodontal membranes of Pg-LPS injected rats were obviously increased compared with control group ( $P < 0.01$ ). This result suggested that the enhancement of amount and activity of the two enzymes might be one of the mechanisms of Pg-LPS to activate osteoclasts.

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**牙龈紫质单胞菌脂多糖通过诱生 IL-1、TNF 和 PGE 激活破骨细胞的骨吸收<sup>1</sup>**

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**关键词** 骨吸收; 白细胞介素 1; 肿瘤坏死因子; 前列腺素 E; 破骨细胞; 牙龈紫质单胞菌; 脂多糖类; 牙周炎

**目的:** 研究牙龈紫质单胞菌脂多糖 (Pg-LPS) 诱生 IL-1、TNF、PGE 和激活破骨细胞的活性, 了解破骨细胞激活机制。 **方法:** 用酚水法制备 Pg-LPS, 采用层析法分离 Pg-LPS 诱生的 IL-1、TNF 和 PGE, 用原子吸收光谱法测定 Ca<sup>2+</sup> 离子浓度, 用组织化学法检测牙周膜中酸性磷酸酶和碳酸酐酶。 **结果:** Pg-LPS 能刺激人外周血单个核细胞 (PBMC) 或人牙周组织细胞分泌 IL-1、TNF 和 PGE。 这些细胞因子的产量在一定范围内随 Pg-LPS 浓度 (0.5-5.0 mg/L) 增高而增加。 IL-1、TNF 和 PGE 能促进 SD 大鼠头盖骨 Ca<sup>2+</sup> 离子的释放, 但以 PGE 的活性最强。 Pg-LPS 注射的 SD 大鼠牙周膜中酸性磷酸酶和碳酸酐酶数量明显升高 (P < 0.01)。 SD 大鼠牙周膜中活化的破骨细胞数量随 Pg-LPS 注射次数增加而明显增多 (P < 0.01), 但由于未活化破骨细胞数量也随之增多而使破骨细胞活化率稳定在 65% 左右。 **结论:** Pg-LPS 具有很强的诱导人 PBMC 和人牙周组织细胞产生 IL-1、TNF 或 PGE 的作用, 这种作用在一定的范围内呈剂量依赖型。 Pg-LPS 可有效地激活破骨细胞, 其激活机制可能与碱性磷酸酶和碳酸酐酶增多有关。

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