Periodontitis is a destructive infectious disease characterized by the loss of periodontal tissue and resorption of the alveolar bone. Porphyromonas gingivalis has been considered to be one of the important pathogenic microorganisms associated with periodontal disease, especially in patients with adult periodontitis. The virulence of this pathogen is attributed to its many cell wall components, especially lipopolysaccharides (LPS). Numerous studies have shown that LPS binds to host cells, such as monocytes, thereby inducing bone resorption.

The Role of Interleukin-1β in *Porphyromonas Gingivalis* Lipopolysaccharide-Induced Bone Resorption

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**Key Words**
bone resorption; lipopolysaccharide; IL-1β converting enzyme

**Background.** Pro-Interleukin (IL)-1β is not fully active after secretion when cleaved with a specific intracellular protease, IL-1β converting enzyme (ICE). ICE-deficient mice display impaired production of IL-1β. Upon stimulation with lipopolysaccharide (LPS), ICE homozygous deficiency mutants are highly resistant to endotoxic shock. Lipopolysaccharides have also been identified as major bacterial factors in bone resorption. However, the exact mechanisms by which LPS stimulates bone resorption in ICE-deficient animals are still uncertain. The present study investigated how interleukin-1β activity mediates LPS-induced bone resorption in vivo.

**Methods.** In our dose-dependent experiment, 10 six-week-old ICE-deficient mice were divided into two groups and injected with one of two concentrations of *P. gingivalis* (A7436) LPS: 500 µg and 100 µg. Ten wild-type mice (164BBC) served as the control subjects. Frozen sections of calvaria were stained with tartrate-resistant acid phosphatase (TRAP) for histomorphometric analysis to quantify osteoclast number, area, and activity.

**Results.** Inhibition of 38% in osteoclast index and osteoclast surface was observed in 500 µg LPS-induced ICE-deficient mice compared with the wild-type mice. However, the data were not significantly different between these two groups at 100 µg of LPS injection, but they were still higher than those of control subjects. Bone-resorbing activities of the osteoclast showed no significant differences between ICE-deficient mice and wild-type mice.

**Conclusions.** Within limitations of the study, we suggest that LPS-induced bone resorption may be caused by enhancing osteoclastogenesis, not by increasing osteoclast resorbing activity. The LPS leading bone resorption is, at least, partially mediated by IL-1β at higher concentrations. [Chin Med J (Taipei) 2002;65:225-230]
macrophages, and fibroblasts, and exerts its biological effects through a cell surface molecule known as the CD14 receptor. This pro cess also requires the presence of LPS binding pro tein (LBP) se creted by the liver. The con se quence of LPS stim u la tion elicits an in flam matory re sponse as well as an im mune host re sponse that can ul ti mately lead to sys temic shock.\textsuperscript{2,3} 

Lo cal re ac tions to endo tox ins are also char ac ter ized by in flam matory re sponse and im mune re sponse. More spec ific ally, vas cu lar changes oc cur, which are ac com pa nied by re cruit ment of leu ko cy tes and the sub sequent re lease of proinflam matory me di a tors, such as pros ta gl din, leu ko tri enes, and cytokines.\textsuperscript{4,5} It is believed that proinflam matory cyto kines, such as TNF, IL-6, IL-8 and IL-1, are the major patho log i cal me di a tors of in flam matory dis eases rang ing from ar thi tis to period on dal dis ease.\textsuperscript{6,7}

Endo tox in or LPS have also been iden ti fied as ma jor bac te rial fac tors in bone re sorp tion, but the ex act mecha nism of ac tion is un der in vest ig ation. Pre vi ous stud ies have de mon strated that LPS stim u la tes osteoclast ac tive bone re sorp tion in di rectly be cause iso lated os teo clasts cul tured on dentine slices and in cu bated with LPS failed to show any resorp tive ac tiv ity.\textsuperscript{8-10} How ever, it is pos si ble that LPS in du ces osteoclast act ivity in di rectly by first stim u lat ing other cell types such as os teo blasts. In the peri od on tum, LPS could cause tis sue dam age through the in duc tion of cyto kines and pros ta glan din s by gingival and period on tal cells. For ex ample, Takada et al. showed that in duc tion of IL-1 and IL-6 oc curred in hu man gingival fi broblast cul tures fol low ing stim u la tion with \textit{P. gingivalis} LPS.\textsuperscript{11} Fur ther more, syn the sis of IL-1, TNF, and IL-6 has been de mon strated in hu man gingival fi bro blasts, and these cytokines have also been de tected in high con cen tra tions in the gingival cre vi cle fluid of period on tal pa tients. The patho genic ro les of these cy to kines in period on dal dis ease have been de mon strated in se veral stud ies.\textsuperscript{12-14}

The two forms of IL-1 gene prod ucts, IL-1\textit{α} and IL-1\textit{β}, are in tra cel lularly fully ac tive, how ever, proIL-1\textit{α} is intracellularly fully ac tive, how ever, proIL-1\textit{β} is processed by a specific in tra cel lular pro te ase called IL-1\textit{β} con vert ing en zyme (ICE) and is usu ally se creted af ter cleav age as a fully mature ac tive mole cule.

Al though the osteolytic and pro-inflam matory prop erties of LPS have been well es tab lished and mice de ficient in the IL-1\textit{β} con vert ing en zyme (lack ing sol uble IL-1\textit{β}) have been shown to be less re spon sive to endo toxin shock,\textsuperscript{15} the role of IL-1\textit{β} in the \textit{in vivo} re sponse to period on dal patho genic LPS has not been well clar i fied. Fur ther more, the stage of osteoclast de vel op ment in flu enced by LPS is not cer tain. There fore, we in vest ig ated the ef fects of \textit{P. gingivalis} LPS-induced bone re sorp tion and osteoclast ac tivity in ICE-deficient mice and wild-type mice as con trols.

\section*{Methods}

For the evalua tion of the role of IL-1\textit{β} on LPS-induced bone re sorp tion and osteoclast dif fer en -
tion, wild-type mice and mice with tar geted de le -
tion of ICE were in jected with LPS pu ri fi ed from \textit{P. gingivalis}.

\subsection*{Mice and LPS in jec tion}

In this ex per i ment, 10 male, 6-week-old, ICE-deficient mice (pro vided by BASF Corp, USA) and 10 wild-type mice (164BBC) were di vided into two groups. Each group, 5 mice, was given a local in jec tion of 500 \textmu g or 100 \textmu g of \textit{P. gingivalis} LPS to the cal varia. Lipopoly saccharides were pre pared from the \textit{P. gingivalis} strain (A7436) us ing a mod ifica tion of the pro ce du re de scribed by West phal et al.\textsuperscript{16} Highly pu ri fi ed LPS were re sus pend ed in 100\textmu l ster ile sa line solu tion us ing 30 sec onds of sonic ation. All ani mals were an es the zed with a ket a mine-xylazine so lu tion (a com bi na tion of 1 mL of ket a mine, 1 mL of xylazine and 6 mL of ster ile PBS) in tramus cular ly . The dose of an es the zed was ad min is tered about 5 \textmu L of an es the zed per gram of body weight. The single-dose injec tions were ad min is tered using a 30/2-ga uge needle at a point lo cated be tween the ears and eyes on the midline of the skull by po sterior-anterior di rection. Five wild-type mice were in jected with 100\textmu L of 0.9\% sa line as the neg a tive con trol sub jects.
Preparation of tissue

Five days following the injections, the mice were humanely killed in a CO\textsubscript{2} chamber. The entire calvaria excluding the soft tissue was dissected and fixed in 4\% paraformaldehyde for 4 hours at 4 °C. Calvarial bones were decalcified with 15\% glycerol in 15\% EDTA solution, pH 7.1 for 21 days at 4 °C. Following decalcification, the specimens were stored in 30\% sucrose over night and then stored in -80 °C pre-chilled 2-methylbutane, ready to section.

Histochemical staining

The calvarial bones were sectioned in half through their sagittal suture and were embedded perpendicularly with OCT compound (Miles Inc., Elkhart, Indiana, USA). Frozen transverse 5 µm serial sections were cut. For each specimen, 50 slides were obtained and every tenth slide was kept for staining. Sections were stained with tartrate-resistant acid phosphatase (TRAP, osteoclast-staining marker). Slides were incubated for 5 min in the staining solution at 37 °C in the dark. Afterwards, the slides were washed with water for 30 min, and then they were counter stained with H&E for 6 min.

Bone histomorphometry

Four slides of each animal, each slide containing two sections, were analyzed. For each tissue section, the two areas with the most abundant resorption area and near calvarial suture were studied at × 200 magnification (magnification). The osteoclast in dex and osteoclast surface were the parameters used to quantify bone re sorption. Os teoclasts were identified at × 400 magnification as multinucleated, TRAP(+) bone lining cells. The osteoclast in index represented the number of os teoclasts per mm of trabecular bone surface (ocs/mm). The osteoclast surface was defined as the percent age of bone surface covered by os teoclasts. This was calculated as the sum of the lengths of each osteoclast containing pit or lacuna (ac tive eroded area) divided by the total trabecular bone perimeter (ocs/Bs) in percentage. These two histomorphometric parameters were measured according to the recommended ASBMR nomenclature.\textsuperscript{17} Bone-resorbing activities of osteoclasts (area resorbed per osteoclast, µm\textsuperscript{2}/oc) were also calculated as the ratio be tween the osteoclastic resorption area (µm\textsuperscript{2}) divided by the number of osteoclasts.\textsuperscript{18}

Statistics

We used the non-parametric Kruskal-Wallis test to compare the differences among the five study groups. We also used the Dunn procedure to compare specific pairs of groups when the results of the Kruskal-Wallis test were significant ($p < 0.01$).

Results

The results of the histomorphometric analysis of the calvarial bones revealed that there were fewer osteoclasts and less bone surface in contact with osteoclasts in mice injected with 100 µg of LPS compared with the 500 µg injected group. In addition, inhibition of 38\% in osteoclast in dex and osteoclast sur face was observed in those ICE-deficient mice that received higher doses of LPS compared with wild-type mice. As expected, the ICE-deficient mice exhibited significantly less osteoclast formation, indicating that IL-1β converting enzyme is important in mediating LPS-induced bone resorption. When 100 µg of LPS was injected, there were no significant differences in bone resorption between the ICE-deficient mice and the wild-type mice, but their bone resorption were still higher than those of saline controls. Moreover, bone-resorbing activity of osteoclasts after 500 µg of LPS in jected was slightly higher than that with 100 µg of LPS, but there were no significant differences ($p > 0.01$) (Table 1).

Discussion

Recent study has shown that alveolar bone loss and periodontal inflammation were greatly reduced when TNF and IL-1 activity were antagonized with func-
tion-blocking soluble receptors.\textsuperscript{19} IL-1 and TNF are cytokines that are synthesized by virtually all human cells, predominantly monocytes and tissue macrophages. Their biological effects overlap extensively, including lymphocyte activation, macrophage activation, prostaglandin formation, cytokine gene expression, endothelial cell activation, and bone resorption.\textsuperscript{4,12}

The biological activity of IL-1 is mediated by two different gene products, IL-1\textalpha{} and IL-1\beta. These two proteins share biological activities and transmit a signal through the same receptor. Both IL-1\textalpha{} and IL-1\beta are synthesized as precursor molecules, proIL-1\textalpha{} and proIL-1\beta. Calpains process proIL-1\textalpha{} to the mature form. Both the pro- and the mature form of IL-1\textalpha{} are biologically active. Whereas proIL-1\beta is markedly less active compared with the mature IL-1\beta, proIL-1\beta is cleaved into its mature and active form by the IL-1\beta-converting enzyme (ICE).

\textit{In vitro} studies have shown that IL-1 causes dramatic increases in osteoclastic bone resorption, and it is probable that the most potent osteolytic cytokine identified so far.\textsuperscript{14,20} IL-1\beta is involved in some pathologic conditions associated with increased bone loss and it is probably the most potent osteolytic cytokine identified so far.\textsuperscript{14,20} IL-1\beta is also important in mediating LPS-stimulated osteoclastogenesis in 500\mu{}g of LPS stimulation, while no statistical differences between mutant and wild-type animals were noted at 100\mu{}g of LPS. Assuma et al. showed that formation of osteoclasts and the amount of bone loss were reduced by 67\% and 60\%, respectively, at the experimental sites with injection of soluble receptors to IL-1 compared with that at the control sites.\textsuperscript{19} These data indicate that higher doses of LPS-induced bone resorption more tends to IL-1 dependence but not lower doses.

Previous studies have shown the induction of responses to LPS in mice lacking TNF receptors and to LPS-induced osteoclastogenesis in transgenic mice lacking type 1 TNF receptor.\textsuperscript{24,25} In addition, osteoclasts formed in the presence of IL-11, a cytokine produced mainly by marrow stromal cells and osteoblasts, were capable of bone resorption and were unaffected by inhibitors of IL-1 and TNF.\textsuperscript{26} More over, the production of IL-1\beta was totally inhibited and the secretion of IL-1\alpha was decreased, but not the serum levels of TNF-\alpha and IL-6, after LPS stimulation in ICE-deficient mice.\textsuperscript{27} Taken together, these suggested that the induction of osteoclast formation and activity by LPS may be through IL-11 or other cytokines as yet unidentified mechanisms at low concentrations of LPS. In addition, the other possible hypothesis is that 100\mu{}g of LPS may be under a triggered threshold of the production of IL-1\beta responsible for bone resorption in wild-type mice. There fore, we suggest the obser vation of the con centration of other cytokines in the serum, which mediates the bone destruction, and the increase of dose-dependent groups for point ing out the threshold of LPS-induced bone resorption. The number of mice may be an other variable. Although bone resorption showed no significant differences at low concentrations of LPS, it still showed a 30\% decrease.

### Table 1. Indices of bone resorption and osteoclast activity in mice injected with \textit{P. gingivalis} lipopolysaccharide and saline

<table>
<thead>
<tr>
<th>Mouse type (No.)</th>
<th>LPS injection amount</th>
<th>Osteoclast index, (ocz/mm)</th>
<th>% Oc surface</th>
<th>Oc activity, area resorbed (\textmu{}m\textsuperscript{2}/oc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICE-deficient (5)</td>
<td>LPS (500 \mu{}g)</td>
<td>4.7 \pm 0.5</td>
<td>20 \pm 2.2</td>
<td>42 \pm 2</td>
</tr>
<tr>
<td>ICE wild-type(5)</td>
<td>LPS (500 \mu{}g)</td>
<td>7.2 \pm 0.6\textsuperscript{a}</td>
<td>32 \pm 1.3\textsuperscript{a}</td>
<td>46 \pm 4</td>
</tr>
<tr>
<td>ICE-deficient (5)</td>
<td>LPS (100 \mu{}g)</td>
<td>2.75 \pm 0.3</td>
<td>10 \pm 0.9</td>
<td>38 \pm 0.9</td>
</tr>
<tr>
<td>ICE wild-type(5)</td>
<td>LPS (100 \mu{}g)</td>
<td>3.47 \pm 0.7</td>
<td>13 \pm 1.3</td>
<td>37 \pm 0.4</td>
</tr>
<tr>
<td>ICE wild-type(5)</td>
<td>0.9% saline</td>
<td>1.0 \pm 0.8</td>
<td>4 \pm 3</td>
<td>38 \pm 3.4</td>
</tr>
</tbody>
</table>

All data are expressed as mean \pm standard error.

\textsuperscript{a}The data are significantly different in osteoclast index, and osteoclast surface compared with the other 4 groups of mice. \textit{P} < 0.01, Kruskal-Wallis test. ICE = IL-1\beta-converting enzyme, oc = osteoclast.
in ICE-deficient mice. This reduced phenomenon may need to increase mouse number for further studying bone resorption in the future.

Study has shown that osteotropic factors can mediate three different stages of osteoclast differentiation: (1) the granulocyte-macrophage colony-forming unit (CFU-GM); (2) mononuclear osteoclast precursors; and (3) mature osteoclasts. One of the bone-resorbing factors is IL-1, which can affect all types of bone resorption as the studies have established as the most potent known in vitro cytokine. Other investigators have demonstrated that IL-1 increased osteoclast precursors and stimulated bone resorption. However, the ratio of area resorbed per osteoclast in IL-1-treated vs control decreased from 5-2-fold at 48-96 hours to 1-fold at 144 hours. In our study, the data showed significantly increased osteoclastogenesis but no significant differences of osteoclast precursors; and (3) mature osteoclasts.

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