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The role of lipopolysaccharide in infectious bone resorption of periapical lesion

Chi-Yuan Hong[1,2], Sze-Kwan Lin[1,2], Sang-Heng Kok[1,2], Shih-Jung Cheng[1], Ming-Shu Lee[2], Tong-Mei Wang[2], Chuan-Shuo Chen[2], Li-Deh Lin[1,2], Juo-Song Wang[1,2]

[1]*School of Dentistry, College of Medicine, National Taiwan University, Taipei, and* [2]*National Taiwan University Hospital, Taipei, Taiwan*

BACKGROUND: The role of lipopolysaccharide (LPS) in periapical lesion-induced bone resorption was investigated. Polymyxin B (PMB), a specific inhibitor of LPS, was evaluated to treat the apical lesion.

METHODS: Lipopolysaccharide isolated from two common endodontic pathogens, *Fusobacterium nucleatum* and *Porphyromonas endodontalis*, stimulated mouse macrophage (J774) to release interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) in a time-dependent manner.

RESULTS: Combination of LPS further enhanced the stimulation. PMB inhibited these effects significantly. LPS also stimulated matrix metalloproteinase-1 (MMP-1) gene expression in J774, whereas anti-IL-1α and anti-TNF-α antibodies, as well as PMB, diminished this effect. A disease model of periapical lesion was established in Wistar rat. Administration of PMB reduced the extent of lesion-associated bone resorption by 76% to approximately 80%, and simultaneously reduced the numbers of MMP-1-producing macrophages.

CONCLUSIONS: It is suggested that LPS released from the infected root canal triggers the synthesis of IL-1α and TNF-α from macrophages. These pro-inflammatory cytokines up-regulate the production of MMP-1 by macrophages to promote periapical bone resorption.

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Keywords: bone resorption; lipopolysaccharide (LPS); macrophage; periapical lesion; polymyxin B

Introduction

Periapical lesions begin as bacterial infection in the dental pulp that subsequently lead to inflammatory bone resorption. Accumulation of bacterial components, such as lipopolysaccharide (LPS) in an infected area, can stimulate the release of pro-inflammatory cytokines from neutrophils and monocytes/macrophages (1). Pro-inflammatory cytokines such as interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) will initiate and augment subsequent inflammatory cascades leading to tissue destruction (2). Recently, large amounts of IL-1α and TNF-α have been detected in induced rat pulpitis (3). These findings highlight the importance of pro-inflammatory cytokines in the pathogenesis of pulpal/periapical disease.

During bone resorption, matrix metalloproteinase-1 (MMP-1) serves primarily for degrading non-mineralized extracellular matrix (4, 5). Stimulation of osteoclastogenesis by MMP-1 through generating collagen-degradation fragments on bone surfaces has also been proposed (6). We have previously demonstrated pronounced expression of MMP-1 gene in macrophages, both in radicular cyst and induced rat periapical lesions (7, 8). These cells aggregated in the vicinity of bone resorption areas and the numbers of MMP-1-expressing cells increased consistently as the lesion expanded, implying the involvement of MMP-1-producing macrophages in the development of periapical lesions (7, 8).

The connection between LPS and the development of periapical lesions and their concomitant bone resorption remains controversial. A positive correlation between LPS and the presence of periapical lesions has been reported (9). The levels of LPS in rat periapical lesions increased with time after lesion induction (10). Direct application of LPS to dental pulp also induced prominent periapical bone destruction in dogs (11). However, some authors argued about the significance of LPS in periapical lesion-induced bone loss (12, 13) in that extracts from periapical lesions elicited obvious *in vitro* bone resorption, whereas LPS inhibitor could not alleviate this osteolytic activity (12).

Great diversity also exists concerning the influence of combined bacterial infection on inflammation. Fabricius et al. (14) reported that infection of monkey root canals with mixed oral bacteria containing *Fusobacterium* and *Bacteroides* induced more advanced periapical bone destruction than when incubated with either strain alone. Conversely, Magnuson et al. (15) found an inhibition of *Bacteroides fragilis* LPS on *Eecherichia coli* LPS-stimulated human endothelial cell adhesiveness for neutrophil.

Correspondence: Dr Juo-Song Wang, School of Dentistry, National Taiwan University Hospital, 1, Chang-Te Street, Taipei 100, Taiwan. Tel.: +886 2 23123456/ext. 6864. Fax: +886 2 23831346. E-mail: jasonw@ntumc.org
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Polymyxin B (PMB) is a cationic peptide with potent antiendotoxin activity (16). It binds to the anionic lipid A, the principal active moiety of LPS, and inhibits most of its activities (17). PMB decreased mortality in a rat model of septic shock and significantly reduced the plasma levels of endotoxin and TNF-α (18). In an experimental system using LPS-stimulated mononuclear cells, after PMB treatment, it was noted that the cells inhibited NF-κB binding activity on DNA and suppressed TNF-α secretion (19).

We hypothesize that LPS released from the infected root canals stimulates macrophages to secrete IL-1α and TNF-α, which, in turn, induce the synthesis of MMP-1 and subsequently, bone resorption. In this study, we investigated the *in vitro* effect of LPS isolated from two common endodontic pathogens on the production of IL-1α and TNF-α by macrophages. Although several kinds of LPS have been reported to stimulate IL-1α and TNF-α production by macrophages, we still have to confirm this response in our study model first before preceding to the subsequent experiments. To elucidate the influence of combined infection, the synergistic or antagonistic interaction between these two LPS were also explored. Furthermore, MMP-1 gene expression in LPS-stimulated macrophage was examined and the modulation of IL-1α and TNF-α in MMP-1 expression was analyzed. Finally, to evaluate the *in vivo* role of LPS on periapical lesions, we compared the effects between normal saline and LPS inhibitor PMB on the progression of induced rat apical periodontitis.

Materials and methods

Materials

Fusobacterium nucleatum (ATCC 25586) and *Porphyromonas endodontalis* (ATCC 25611) were purchased from American Type Culture Collection (Manassas, VA, USA). PMB was purchased from Sigma (St Louis, MO, USA). Anti-IL-1α and anti-TNF-α antibodies were purchased from Serotec (Oxford, UK). Mouse IL-1α, IL-1β, and TNF-α ELISA kits were purchased from R&D (Minneapolis, MN, USA).

LPS extraction

Fusobacterium nucleatum and *P. endodontalis*, the essential anaerobic Gram-negative pathogens of endodontic lesion (9), were grown under anerobic conditions and harvested at the end of the logarithmic phase of growth. LPS was purified following the hot phenol–water method of Westphal with few modifications (20). The endotoxin level of the LPS was assayed by limulus ameobocyte lysate (LAL) test which showed that LPS from *F. nucleatum* and *P. endodontalis* contained 1.0 ± 0.23 endotoxin unit (EU)/μg and 0.09 ± 0.01 EU/μg, respectively.

Cell culture

J774, a mouse monocyte/macrophage cell line, was maintained in RPMI 1640 media supplemented with 10% fetal calf serum containing less than 0.1 EU/ml of endotoxin, 2-mercaptoethanol, antibiotics, amphotericin B, and HSPES. Serum was pre-treated by heating to 56°C for 45 min to destroy complement factors.

Analysis of cytokines released into the culture media

The amounts of TNF-α, IL-1β, and IL-1α released to the culture media following LPS stimulation were analyzed by enzyme-linked immunosorbent assay (ELISA; R&D, Minneapolis, MN, USA). Briefly, standard or sample solution was added to ELISA well plate, which had been pre-coated with specific monoclonal capture antibody. After shaking gently for 3 h at room temperature, polyclonal anti-TNF-α, IL-1β, or IL-1α antibody, conjugated with horseradish peroxidase, was added to the solution, respectively, and incubated for 1 h at room temperature. Substrate solution containing hydrogen peroxidase and chromogen was added and allowed to react for 20 min. The levels of cytokines were assessed by a microelisa reader at 450 nm and normalized with the abundance of standard solution. Each densitometric value expressed as mean ± SD was obtained from three independent experiments.

Northern analysis

Total RNA was isolated using the acid guanidium thiocyanate–phenol–chloroform method (Zol-B, Biotecx, Houston, TX, USA), electrophoresed and transferred to a nylon membrane. RNA was immobilized by ultraviolet cross-linking. The membrane was hybridized with radiolabeled cDNA probes for murine MMP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; as internal standard) and then washed under high-stringency conditions followed by autoradiography at −80°C. The intensity of each band, after normalization with GAPDH mRNA, was quantified by a scanning video densitometer and software (Biomed Instrument, Fullerton, CA, USA). Each densitometric value, expressed as mean ± SD, was obtained from three independent experiments.

In vivo *effect of PMB on induced rat periapical lesion*

Periapical lesions were induced in 44-week-old Wistar rats as described in our previous reports (8, 21, 22). The experimental protocol was approved by the Experimental Animal Center, National Taiwan University, and the animals were maintained following the Guide to Management and Use of Experimental Animals, National Science Council, Taiwan. At day 0, the animals were anesthetized by intraperitoneal injection of 3 mg of sodium pentobarbital per 100 g body weight and mounted on an operating table. Pulp exposure was performed at the distal fossa of right mandibular first molars using #1/4 round bur to the depth of bur diameter. The exposed pulps were left open to the oral environment to induce the formation of periapical lesions. One day before pulp exposure, the rats (10 per group) were given intraperitoneal injections of either PMB (0.25, 1, and 2 μg/g body weight) or normal saline (NS; as control) on a daily basis until sacrifice. Body weights were measured every 3 days for dose adjustment. Adverse tissue response was carefully monitored. The animals were sacrificed 20 days after pulp exposure, which is the maximal lesion expansion period (21).

Image analysis

The jaws were dissected and processed for image analysis as described in our previous reports (8, 21, 22). Briefly, radiographs were taken by a microradiograph device and ana-

lyzed by a Digora image analysis system (Soredex, Helsinki, Finland). The areas of periapical lesions at the distal root apices of the right mandibular first molars were quantified in pixels using a specially designed software. Data were transformed to square millimeters using $1\,mm^2 = 256$ pixels, as determined by assaying a standard of known area.

Immunohistochemistry

After image analysis, the mandibles were fixed, decalcified, and embedded in paraffin. Periapical lesions associated with the surrounding bone tissue were prepared in 5-μm serial sections. Immunohistochemical staining was performed using a streptavidin–biotin method. Monoclonal antibodies of murine antihuman/rat MMP-1 (Oncogen Science, Cambridge, MA, USA) and murine antiratmonocyte/macrophage lysosomal membrane (ED-1, Serotec, Oxford, UK, for identifying macrophages; 23) were used to detect the target molecules or cells within periapical lesions.

Cell count

For each periapical lesion, quantitative analysis was performed on three sections. Only those sections containing root apex with periapical lesion in the center were selected. The field in each section exhibiting strongest MMP-1 synthetic activity was selected and examined under microscopic high power field (HPF) at ×300 magnification. In total, three HPFs were analyzed for each lesion and 30 HPFs (three HPFs/lesion × 10 lesions/group) were examined in each group. Each HPF contained at least 250 cells. Macrophages were identified as large mononuclear cells with positive ED-1 staining. Because ED-1 is also reactive with osteoclasts, the multinucleated giant cells (osteoclasts) in Howship's lacunae were excluded from the enumeration. Two countings were taken: the number of MMP-1-producing macrophages and the total number of cells examined. The data were converted to the percentage of MMP-1-producing macrophages/HPF.

Statistical analysis

Data were subjected to ANOVA analysis for multiple comparisons and then Fisher's protected least significant difference test. $P < 0.05$ was considered statistically significant.

Results

LPS stimulated IL-1α and TNF-α synthesis in macrophages

After treatment with *F. nucleatum* and *P. endodontalis* LPS, culture media of J774 were collected and analyzed by ELISA for TNF-α and IL-1α production. The *F. nucleatum*, *P. endodontalis* LPS (0.1 EU/ml), either alone or in

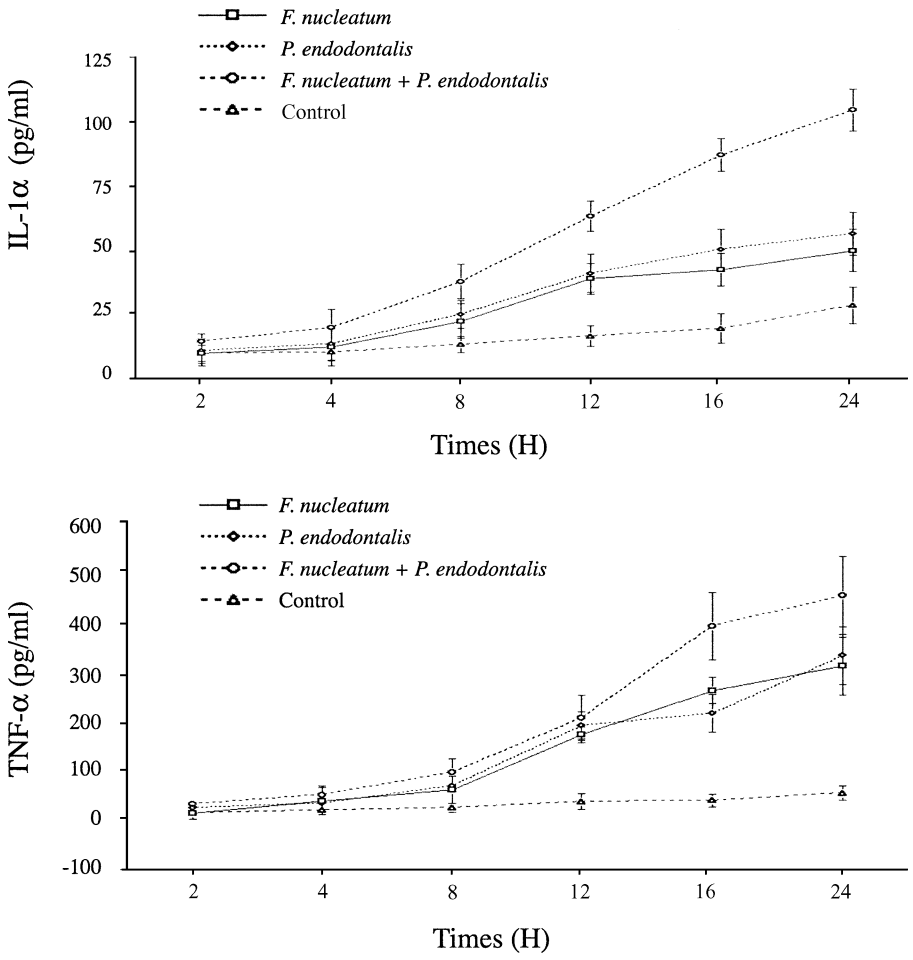

Figure 1 Time-dependent production of IL-1α and TNF-α by J774 stimulated by 1 EU/ml of LPS from *F. nucleatum* (□), *P. endodontalis* (◇), or in combination (○) and control (△). Each bar represents mean ± SD of three independent experiments.

Table 1 Effects of PMB (0.25, 1, 2 μg/g body weight) on the size and percentage of MMP-1$^+$ macrophage in periapical lesion by day 20

	NS	*PMB (0.25)*	*PMB (1)*	*PMB (2)*
Lesion Size (mm^2)	0.81 ± 0.089	$0.19 \pm 0.027^*$	$0.18 \pm 0.036^*$	$0.17 \pm 0.024^*$
Percentage of MMP-1$^+$ macrophage‡	39.2 ± 9.8			$10.8 \pm 2.1^*$

$^*P < 0.05$ vs. NS group.
‡Mean ± SD per high power field.

combination, stimulated the production of IL-1α and TNF-α in a time-dependent manner (Fig. 1). However, the inductive effect on IL-1β synthesis was inconclusive (data not shown). LPS from the two bacteria on an identical EU basis exhibited similar potency in triggering cytokine production. Furthermore, compared with *F. nucleatum* or *P. endodontalis* LPS alone, combination of LPS exhibited an addictive outcome with regard to IL-1α and TNF-α production. The effect became more obvious after incubation of LPS for 16–24 h.

Effects of PMB on LPS-induced cytokine production

Polymyxin B alleviated the LPS-induced synthesis of IL-1α and TNF-α in a dose-dependent manner. At 1 μg/ml, PMB reduced the *F. nucleatum* LPS (1 EU/ml)-stimulated 24-h synthesis of IL-1α and TNF-α by 39 and 58%, respectively (Fig. 2). The inhibition of IL-1α and TNF-α production by 10 μg/ml PMB was 60 and 70%, respectively. PMB also abolished the cytokine-inductive effects of *P. endodontalis* LPS (data not shown).

PMB and cytokine antibodies inhibited LPS-stimulated MMP-1 gene expression in macrophages

Cells were treated with LPS of various concentrations (1, 5, and 10 EU/ml) for 24 h. Transcription of MMP-1 mRNA was assessed by Northern analysis. The results showed that LPS-induced MMP-1 gene expression (Fig. 3A) and the stimulatory effect was dose-dependent (Fig. 3B). Furthermore, PMB (10 μg/ml) and anti-IL-1α, anti-TNF-α antibodies (10 μg/ml) blocked the LPS-induced MMP-1 expression markedly (Fig. 3C,D).

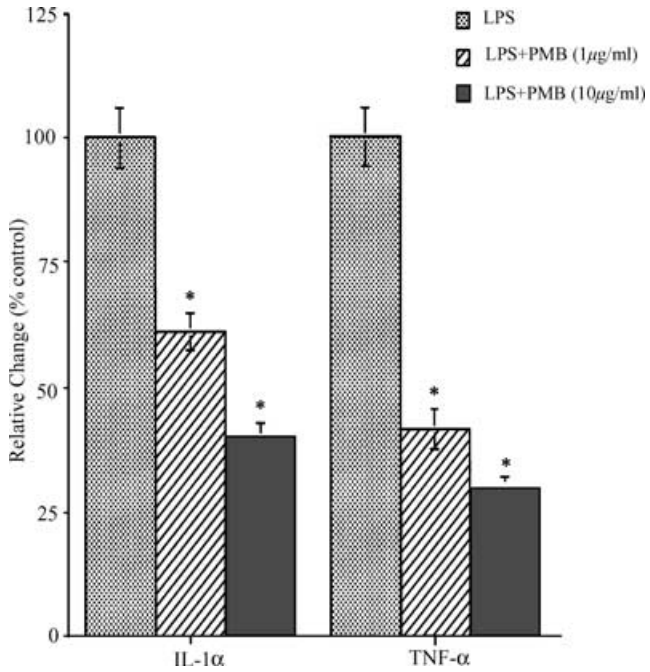

Figure 2 Effects of PMB on IL-1α and TNF-α synthesis following *F. nucleatum* LPS (1 EU/ml) stimulation for 24 h. Each bar represents mean ± SD of three independent experiments. $^*P < 0.05$ vs. LPS alone.

PMB inhibited the progression of induced rat periapical lesion

In the induced periapical lesions, ED-1-positive cells were identified as round, oval, or dendritic cells with dark brown granules within the cytoplasm. Most of the mononuclear ED-1-positive macrophages aggregated around the root apex and in the areas showing active bone resorption (Fig. 4A,B). Prominent MMP-1 was identified in these ED-1-positive cells (Fig. 4C). One day before pulp exposure, the rats were injected with normal saline (NS; as control) or PMB (0.25, 1, and 2 μg/g body weight) once daily until sacrifice at day 20. Image analysis revealed that PMB effectively inhibited the periapical lesion-induced bone resorption by 76–80% (Fig. 5, Table 1). No significant difference was found between the effects of the three concentrations of PMB administered (Table 1). Marked reduction in the percentage of MMP-1-producing macrophages after PMB (2 μg/g body weight) treatment was also noted (Fig. 4D, Table 1).

Discussion

The term 'LPS' does not denote a single molecular species, but rather molecules of heterogeneous chain lengths. For instance, the *Bacteroides* LPS appears as a series of molecules with high and low molecular weights in SDS–PAGE (24). Extraction method greatly affects the biological activity of LPS (24). Among the three extraction methods – the phenol–chloroform method (25), the triton–Mg^{2+} method (26), and the aqueous phenol method (20) – the last one can obtain LPS with highest activity in terms of cytokine induction (24). We found that on an identical EU basis, LPS from *P. endodontalis* and *F. nucleatum* exhibited similar biological activity. These findings support the use of EU as LPS unit, at least in the assay of cytokine expression.

A previous study of periapical lesion has demonstrated that IL-1α and TNF-α, produced primarily from macrophages, are related with the progression of bone loss (3). In the present study, LPS from *F. nucleatum* and *P. endodontalis* elicited the secretion of IL-1α and TNF-α from J774 in a time-dependent manner. LPS also up-regulated MMP-1 gene expression, whereas neutralizing anti-TNF-α and anti-IL-1α antibodies abolished these effects significantly. Recently, we have demonstrated that in rat periapical lesions, macrophages are the main cellular source of MMP-1 during the active phase of lesion development. These cells tend to gather around areas of active bone

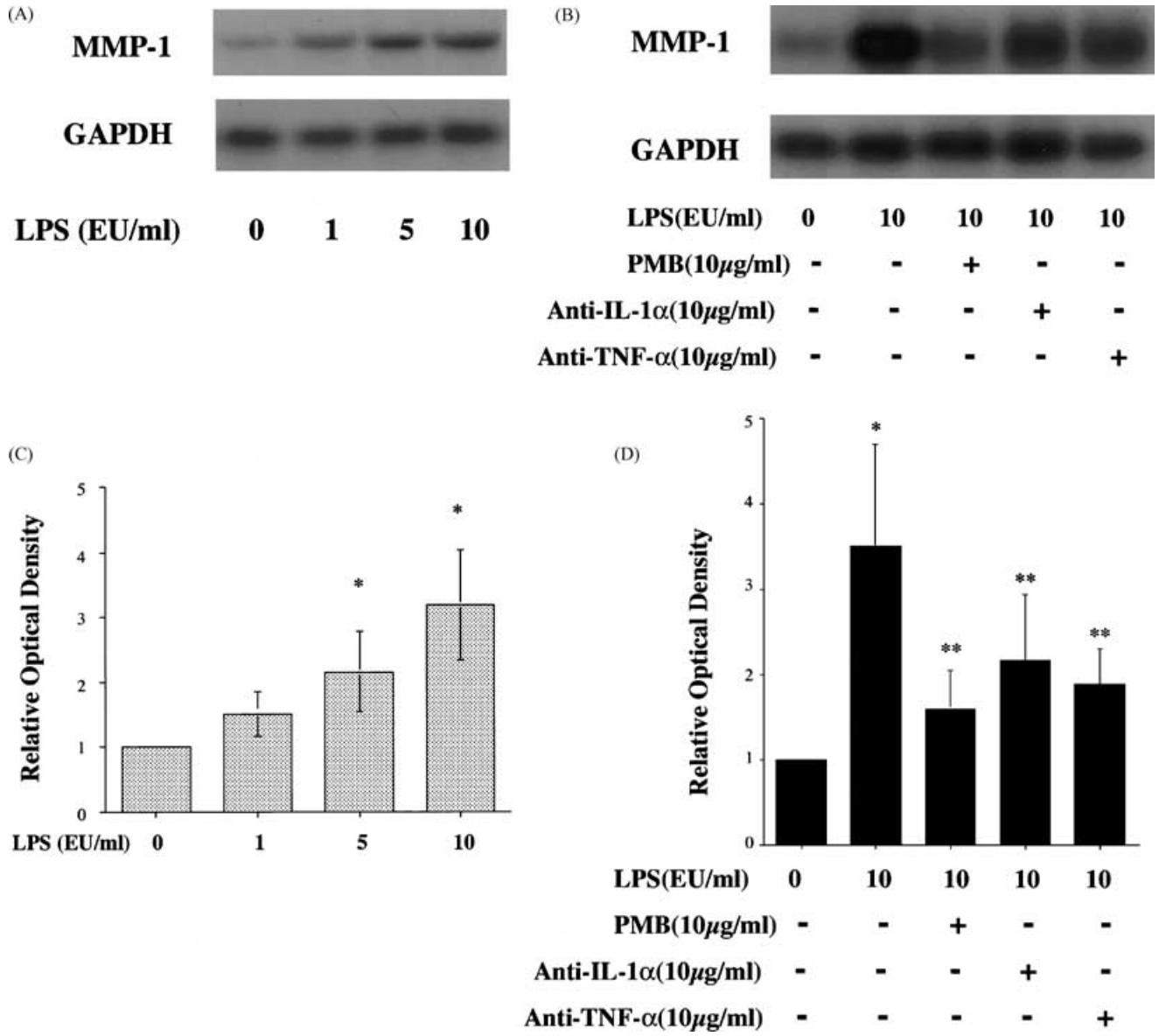

Figure 3 Expression of MMP-1 gene in J774 in response to LPS treatment (A,B). The mRNA levels of MMP-1 were analyzed after 24 h stimulation. Note that LPS induced MMP-1 gene expression dose-dependently (C,D). Anti-IL-1α, anti-TNF-α antibodies (10 μg/ml) and PMB (10 μg/ml) attenuated this effect. $^{*}P < 0.05$ vs. control, $^{**}P < 0.05$ vs. LPS alone. Each bar represents mean ± SD of three independent experiments.

resorption and their numbers increase consistently as the lesions expand (8). PMB, a cationic peptide with potent antiendotoxin activity, abolished the synthesis of IL-1α and TNF-α from J774 stimulated by LPS. PMB also inhibited the LPS-stimulated MMP-1 gene expression. Furthermore, in our rat model of periapical lesion, administration of PMB reduced the extent of lesion-associated bone loss as well as the amounts of MMP-1-synthesizing macrophages. Taken together, it is suggested that LPS released from the infected root canal triggers the production of IL-1α and TNF-α from macrophages. These pro-inflammatory cytokines modulate the subsequent production of MMP-1 from macrophages to promote periapical bone resorption.

ELISA showed that LPS from *F. nucleatum* and *P. endodontalis* enhanced each other's effect. This may partially explain the findings by Fabricius et al. (14) that combined bacterial infection of root canals induced more severe periapical bone loss than infected with either strain alone. Our findings imply an addictive effect between mixed bacterial infection on the progression of periapical lesions, at least with regard to the induction of pro-inflammatory cytokines. Furthermore, as infected root canals are composed of a mixed population of bacteria (9, 27), the *in vivo* interactions between different bacterial components in periapical lesions may be far more complex than our *in vitro* model. Conversely, *B. fragilis* LPS diminished significantly the *E. coli* LPS-stimulated endothelial cell adhesiveness for neutrophil (15). Whether these diverse phenomena were because of the presence of CD14 in target cell or not deserve further investigation, as this important cell surface LPS-binding site is expressed primarily in myeloid cells but not in endothelial cells (28).

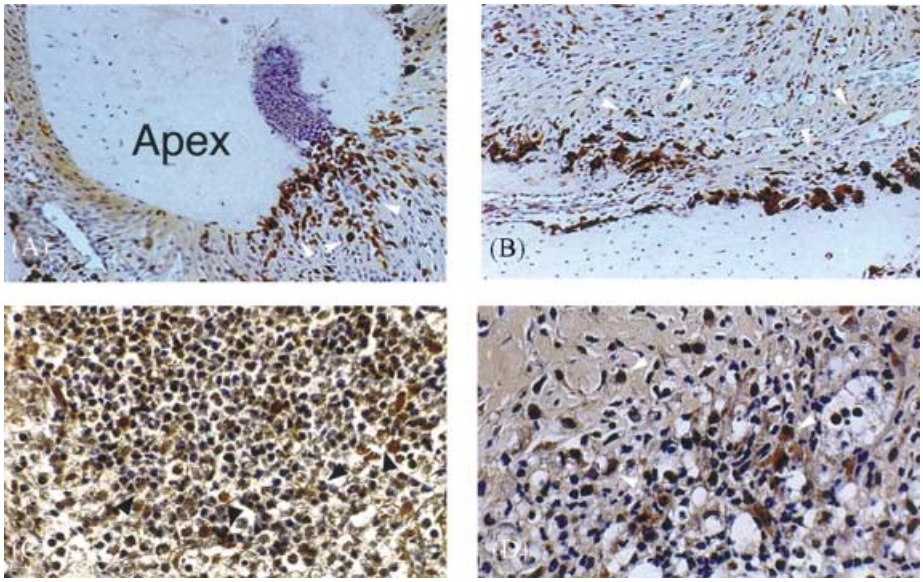

Figure 4 Immunolocalization of ED-1 and MMP-1 in rat periapical lesions. Macrophage marker ED-1 is identified in mononuclear round cells (arrowheads) around the root apex (A) and osteolytic areas (B,C). Pronounced MMP-1 synthesis is found in mononuclear round cells (arrowheads; D). Administration of PMB diminished the numbers of MMP-1^{+} macrophages (arrowheads; A and B, $\times$150; C and D $\times$300).

Our experiment demonstrated that PMB reduced the size of induced rat periapical lesions by 76–80%. This is in accord with the results of earlier studies, which showed a positive correlation between LPS level and severity of infectious pulpal/periapical disease (29, 30). However, some investigators found that extracts from periapical lesions in rapid bone destruction stage elicited obvious *in vitro* bone resorption, but co-incubating with PMB did not alleviate the osteolytic activity (12). This is because LPS induces bone resorption by stimulating the release of osteolytic mediators, such as IL-1α and TNF-α, rather than acting directly on the bone. PMB has no protective effect once the inflammatory cascade has been initiated.

In our study, the level of IL-1β synthesized from LPS-stimulated J774 was insignificant compared with the high levels of IL-1α and TNF-α produced. A previous investigation on the expression of pro-inflammatory cytokines in induced rat periapical lesions also demonstrated high levels of IL-1α and TNF-α mRNAs, but only very slight amount of IL-1β mRNA (31). These findings, as well as the results from the present study, exclude the role of IL-1β in the pathogenesis of rat periapical lesions. In contrast, significant

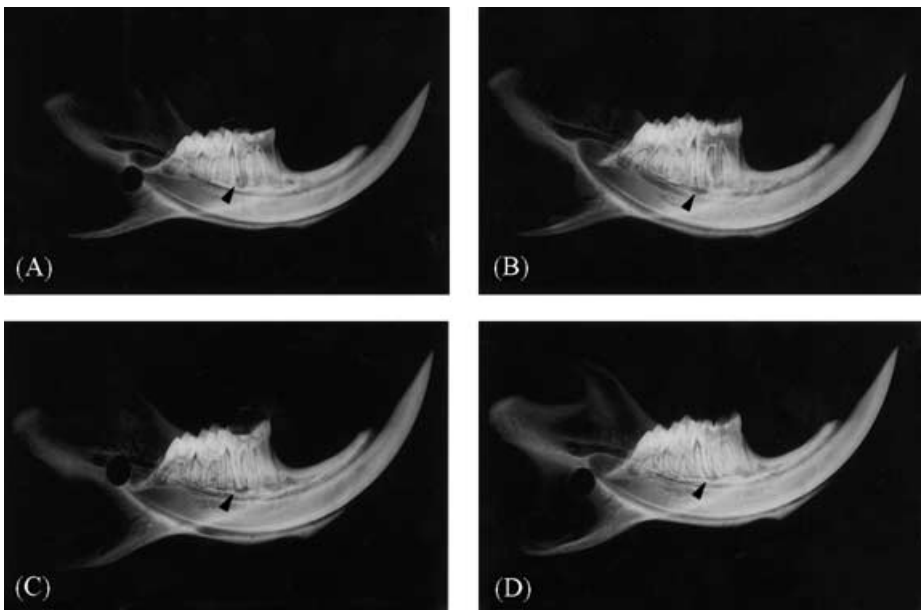

Figure 5 Polymyxin B diminished periapical lesion-associated bone resorption. The jaws were dissected at day 20 after pulp exposure. Radiographs were taken to measure the areas of periapical lesions at the distal root apices of the right mandibular first molar (arrowheads). (A–D) are animals receiving normal saline, 0.25, 1, and 2 μg PMB/g body weight, respectively. Note that PMB retarded the progression of periapical lesions.

level of IL-1β mRNA was identified in macrophages in human radicular cysts (32). Species variation may be a possible explanation.

In addition to IL-1α and TNF-α, our recent study demonstrated that LPS from *F. nucleatum* also induced the expression of inducible nitric oxide synthase (iNOS) and transforming growth factor-β1 (TGF-β1) genes in J774 (22). The iNOS or TGF-β1-expressing macrophages also distributed in the vicinity of osteolytic areas in rat periapical lesions (21, 22). It appears that LPS can promote bone resorption via triggering macrophages to release a host of osteolytic mediators. However, it should be noted that LPS is not the only bacterial component capable of inducing bone resorption (33). In fact, non-LPS components, such as lipoprotein, peptidoglycan, capsular polysaccharides, either from Gram-positive or Gram-negative strains, have also been reported to stimulate the release of bone-resorptive cytokines (34, 35).

In summary, we do not know exactly whether *F. nucleatum* and *P. endodontalis* exist in rat periapical lesion because of the scarcity in bacteriological information. Our findings still support the view that LPS plays a significant role in the pathogenesis of periapical lesions. It triggers the inflammatory osteolysis by inducing the release of pro-inflammatory cytokines and MMP-1 from macrophages. However, it should be noted that the macrophages accumulating around the bone resorption area may also serve as osteoclast progenitor cells itself in addition to producing osteoclast-stimulating factors such as IL-1α and TNF-α.

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