IL-1β production and gene expression by peptidoglycan from *Lactobacillus casei* in human dental pulp cells of deciduous teeth and permanent teeth

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Abstract
The purpose of this study was to compare the production of interleukin-1β (IL-1β) by peptidoglycan from *Lactobacillus casei* (*L. casei*) in human pulp-derived fibroblasts from deciduous (DHPF) and permanent teeth (HPF). DHPF and HPF were collected from 6 noncarious deciduous teeth (6-9 year-old individuals) and 6 noncarious permanent teeth (20-25 year old individuals) extracted in the course of orthodontic treatment. Both IL-1β protein production and IL-1β mRNA levels from peptidoglycan-treated DHPF and HPF were clearly enhanced, compared to IL-1β protein production and IL-1β mRNA levels from peptidoglycan-non-treated DHPF and HPF that were not treated by peptidoglycan. Furthermore, IL-1β protein production and IL-1β mRNA levels from peptidoglycan-treated DHPF showed clearly lower levels, compared with IL-1β protein production and IL-1β mRNA levels from peptidoglycan-treated HPF. These findings suggest that pulpitis in deciduous teeth was less induced than pulpitis in permanent teeth, and that *L. casei*-peptidoglycan stimulated IL-1β production through enhancement of IL-1β mRNA expression from DHPF and HPF.

Introduction
It is generally accepted that bacteria do not invade the dental pulp if clinically sound dentine remains beneath the bottom of the carious lesions. However, it has been reported that bacteria can be isolated from deep layers of dentinal lesions where the dentine has not been softened. (1) In addition, microscopic observation has revealed that bacteria advance into dentinal tubules unevenly; i.e., some bacteria as deeper in some individual tubules. (2) Thus, it is possible that in teeth with no clinical evidence of pulpal exposure and with pulp covered by clinically sound dentine, that small numbers of bacteria can still invade the dental pulp through only a few specific dentinal tubules. (3)

In the microbiology of deep carious lesions, lactobacilli have received more attention as participants. (4) Strict anaerobic cultures of microorganisms from deep carious lesions recover mostly lactobacilli, with only small amounts of streptococci. (5) Furthermore, in pulp tissues with carious lesions or bacterial infections, lactobacilli are the most commonly isolated microorganisms, and with lactobacilli, the bacterium chiefly isolated is *Lactobacillus casei* (*L. casei*). (4, 5)

In the dental pulp response stimulated by peptidoglycan, which was extracted from *L. casei* cells, it has been reported that peptidoglycan from *L. casei* induces inflammatory cell infiltration in the dental pulp subjacent to cut dentinal tubules. (6) Furthermore, there has been a report in the literature describing peptidoglycan from *L. casei* as one of the peptidoglycans extracted from mainly isolated microorganisms in carious lesions and as a stimulant for prostaglandin E₂ (PGE₂) production from human-pulp-derived fibroblasts of permanent teeth (HPF). (7) This finding indicates that peptidoglycan extracted from *L. casei* in microorganisms of carious lesions stimulated PGE₂ production from HPF most strongly. PGE₂ has been strongly implicated in pulpal inflammation, particularly vascular permeability. (8) Hence, the author thought that peptidoglycan from *L. casei* in microorganisms of carious lesions would induce inflammation reaction in HPF most strongly.

Investigations of the inflammation reaction by the inflammatory cytokine, healthy dental pulp, and pulpitis in human teeth, found that IL-1 activity of pulps from carious teeth with pain in permanent teeth is significantly enhanced in comparison to IL-1 activity of pulps from carious teeth without pain in permanent teeth. IL-1 is a proinflammatory...
cytokine and a key component of cellular-immune responses. (9) Furthermore, IL-1 has been reported to enhance interstitial collagenase gene expression from HPF in vitro. (10) Interstitial collagenase is a member of a family of matrix metalloproteinases (MMPs) that play a role in degradation of the extracellular components, such as collagens in normal extracellular matrix remodeling and inflammation processes. Hence, the author considered that IL-1 would be deeply involved in the progress of pulpitis.

On the other hand, the general clinical impression of pediatric dentists, is that deciduous teeth have a lower sensitivity to pain than permanent teeth, which may be due to differences in number and/or distribution of their neural components. (11) However, little is known regarding the effects of inflammatory cytokines in human-pulp-derived fibroblasts of deciduous teeth (DHPF).

Therefore, this study investigated IL-1β production and IL-1β mRNA expression levels from DHPF and HPF stimulated by peptidoglycan isolated from L. casei. The purpose of this study was to compare and assess IL-1β production and IL-1β mRNA expression levels from DHPF and HPF.

**Materials and methods**

1. **Peptidoglycan fraction preparation (PG)**

   *L. casei* (ATCC4646) was cultured in brain heart infusions supplemented with 0.25% yeast extract, 0.01% hemin, and 0.001% vitamin K. The microorganisms were grown at 37 °C in an anaerobic chamber containing 80% N₂, 10% H₂, and 10% CO₂. PG was prepared and purified from *L. casei* cells by the method of Yanai *et al.* (12)

2. **Cell culture**

   DHPFs and HPFs were collected from 6 noncarious deciduous teeth (6-9 year-old individuals) and 6 noncarious permanent teeth (20-25 year old individuals) extracted during orthodontic treatment. The Ethical Committee of the Nihon University School of Dentistry at Matsudo approved the study. All patients were given informed consent before the sample was derived. DHPF and HPF were prepared according to a modification of the method of Somerman *et al.* (13)

   For the experiments, DHPF and HPF were used at passages 6 to 9 and were plated at 5 × 10⁴ cells (0.5 ml medium) per well in 24-well plates. The confluent-stage cells were incubated for 24 h in a medium containing 2% fetal calf serum (FCS). The medium was removed, and the cells were washed twice with PBS, and then cultured for the indicated times at 37 °C in fresh medium containing 2% FCS with or without *L. casei* PG (10 μg/ml). The conditioned media were collected and stored at −80 °C until use.

3. **IL-1β assay**

   The concentration of IL-1β was determined using an Endogen Human IL-1β ELISA Kit (Endogen, Inc).

4. **Isolation of RNA**

   Total cellular RNA was extracted from DHPF and HPF treated with or without PG by RNeasy Mini Kit (QIAGEN). The final RNA precipitate was stored at -135 °C.

5. **RT-PCR**

   cDNA synthesis and amplification by RT-PCR were conducted using the Super Script One-Step RT-PCR System (GibcoBRL). The PCR primers for IL-1β and glyceraldehyde phosphate dehydrogenase (GAPDH) were designed with reference to the reported IL-1β(14) and GAPDH(15) cDNA sequences. As a control, the author amplified GAPDH. The sequences of primers were as follows: 5'-CTA GTG ACT TGA CGT GCG-3' (forward primer for IL-1β); 5'-CAT CAG CAC CTC TCA AGC-3' (reverse primer for IL-1β); 5'-GCG CAG AAT GAG ATG AGT-3' (forward primer for GAPDH); and 5'-ATG GAC TGT CGT CAT GAG-3' (reverse primer for GAPDH). The DNA
fragments were separated by electrophoresis in a 10% polyacrylamide gel. Gels were stained with ethidium bromide and photographed under ultraviolet light.

6. Statistical methods

Results were shown as the mean value ± standard deviation (S.D.). Statistical analysis was performed using the SPSS Ver. 10.0 for windows computer program (SPSS Japan Inc.). Tukey-Kramer test was used to compare the mean values of the groups.

Results

1. Time courses of IL-1β protein production

The time courses of IL-1β protein production in the conditioned medium from DHPF and HPF of each patient incubated for 4, 8, 12 and 24 h with and without 10 μg/ml of regulated L. casei PG were examined (Fig. 1). IL-1β protein production of PG-treated DHPF and HPF showed a significant increase at 8, 12 and 24 h (p < 0.01) compared with PG-non-treated DHPF and HPF. IL-1β protein production from PG-treated DHPF indicated a significant low at 8, 12 and 24 h (p < 0.01) compared to IL-1β protein production from PG-treated HPF.

2. Effects of PG on IL-1β protein production

The effects of PG concentration on IL-1β protein production from DHPF and HPF of each patient treated with and without 0.1, 1 and 10 μg/ml of L. casei PG for 8 h were examined (Fig. 2). IL-1β protein production from PG-treated HPF were significantly enhanced in 1 μg/ml (p < 0.05) and 10 μg/ml (p < 0.01) compared to IL-1β protein production of PG-non-treated HPF. IL-1β protein production from PG-treated DHPF were significantly increased in 10 μg/ml (p < 0.01) compared to IL-1β protein production from PG-non-treated DHPF. IL-1β protein production from PG-treated DHPF showed a significant low in 1 μg/ml (p < 0.05) and 10 μg/ml (p < 0.01) compared to IL-1β protein production of PG-treated HPF.
3. Comparison of IL-1β protein production

IL-1β protein productions of DHPF and HPF of six patients when those cells were incubated with 10 µg/ml of L. casei PG for 8 h are shown in Figs. 3 and 4. IL-1β protein production from PG-treated DHPF and HPF was significantly enhanced compared to IL-1β protein production of PG-non-treated DHPF and HPF. IL-1β protein production of PG-treated DHPF showed a significant low compared to IL-1β protein production of PG-treated HPF.

Statistical analyses of the relationship between DHPF and HPF of the six patients, in terms of PG-treated IL-1β protein production, are summarized in Table 1. Tukey-Kramer’s test revealed that IL-1β production from PG-treated DHPF and HPF was significantly enhanced compared to IL-1β protein production of PG-non-treated DHPF and HPF (p < 0.01). IL-1β protein production from the PG-non-treated DHPF was increased compared to IL-1β protein production of PG-non-treated HPF (p < 0.01). IL-1β protein production of PG-treated HPF showed a significant low compared to IL-1β protein production of PG-treated HPF (p < 0.01).

4. RT-PCR

To identify whether the increase of IL-1β protein was derived from L. casei PG, which induced elevation of IL-1β mRNA expression in the conditioned medium, the author used RT-PCR analysis of total RNA that was isolated from DHPF and HPF of each a patient after incubation with 10 µg/ml of L. casei PG (Fig. 5). For the control, the levels of GAPDH mRNA were found to be similar in all of the cells. The levels of IL-1β mRNA (368 base pairs) obtained from the PG-treated DHPF and HPF were higher than the levels of IL-1β mRNA obtained from PG-non-treated DHPF and

Table 1. Statistical analyses of IL-1β production from HPF and DHPF in the presence (PG+) and absence (PG-) of PG

<table>
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<tr>
<th>IL-1β production</th>
<th>Turkey-Kramer</th>
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<tr>
<td>DHPF (PG-) vs. DHPF (PG+)</td>
<td>&lt;0.01**</td>
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<tr>
<td>HPF (PG-) vs. HPF (PG+)</td>
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<td>DHPF (PG-) vs. HPF (PG-)</td>
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<td>DHPF (PG+) vs. HPF (PG+)</td>
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(n=6, **P<0.01)
The levels of IL-1β mRNA in PG-treated DHPF were showed lower than the levels of IL-1β mRNA of PG-treated HPF.

Discussion

Dental pulp is a unique tissue of ectomesenchymal origin and is enclosed in ringed walls of dentine. The response of the dental pulp from carious lesions involves both specific and nonspecific inflammatory reactions. Nonreversible tissue damage occurs chiefly due to the process of inflammatory response to bacterial invasion.

In the mechanisms of pulp-tissue damage by bacterial irritants, in vitro experiments for studying chemotaxis have shown that migration of inflammatory cells can be mediated not only as a direct result of bacterial substances, but also as an indirect of bacterial activation of the complement system. (17) This system is as a series of effector substances of the host defense with the capacity to initiate and potentiate the inflammatory response. (18) Various bacterial products activate the system as well as antigen-antibody complexes. On activation, the complement system can stimulate chemotaxis of leukocytes and subsequent phagocytosis of foreign agents and antigen-antibody complexes. However, by doing so, attracted inflammatory cells release lysosomal enzymes into the environment with a capacity to degrade not only bacteria and their products, but also connective tissue components. Thus, the pulp-tissue damage may be induced by the development of inflammation due to bacterial substances.

In the degree of dentine caries in caries lesions induced pulptitis, it has been reported that these pulp responses are produced by bacterial and antigenic substances that penetrate through intact but exposed dentin. Histopathological analysis of pulp tissue in teeth with carious lesions indicates that pulpal inflammation manifests itself beneath superficial caries in the carious process before carious lesions have made contact with the pulpal tissue. (19) Hence, it has been reported that this inflammatory reaction in the pulp occurs because of the movement of bacteria and antigenic substances through the dentinal tubules into the pulp. (20)

A study of the microbiology of deep carious lesions reported that the amount of L. casei gradually increases in the deep carious sites and the pulpal surface. (5) Another investigation of the bacteriology on approximal carious plaque and the corresponding dentine lesion found that L. casei is isolated from the carious dentine of two thirds of the lesions. (21) In addition, in an analysis of the colony counts from the underlying carious dentine, L. casei constituted 85 percent of the dentinal isolates with other gram positive rods and streptococci making up 8 and 5 percent respectively. (21) Furthermore, in the dental pulp response stimulated by PG extracted from L. casei cells (L. casei PG), L. casei PG induced inflammatory cell infiltration in the dental pulp subjacent to cut dentinal tubules. (6) Another study of inflammatory cytokine from DHPF or HPF stimulated by L. casei PG, reported that L. casei PG stimulated HPF enhanced production of IL-6, (22) and IL-6 is involved in the final differentiation of B-cells 23) and stimulates acute-phase proteins such as C3. (24) Thus, these findings indicate that L. casei is a bacterium that has the greatest influence on dental pulp and is involved in developing pulpitis. And the author examined this study by using L. casei PG.

On the other hand, as we have already mentioned, the general clinical impression of pediatric dentists is that deciduous teeth may have a lower sensitivity to pain than pain in permanent teeth, which may be due to differences in number and/or distribution of their neural components. (25) However, little is known regarding the effects of inflammatory cytokines in DHPF.

A study of inflammatory cytokines and pulptitis reported that IL-1 activity of pulps from carious teeth with pain is significantly enhanced compared to IL-1 activity of pulps from carious teeth without pain; therefore, IL-1 in human dental pulp indicates the involvement of the mediator of inflammation in dental disease. (26) It is also known that IL-1 enhances interstitial collagenase gene expression from HPF, (27) increases the synthesis of several acute-phase proteins, and activates polymorphonuclear leukocytes (28). To identify a difference in the inflammatory reaction of the pulp between deciduous teeth and permanent teeth, this study examined IL-1β protein production and IL-1β mRNA expression levels from DHPF and HPF stimulated by L. casei PG. The purpose of this study was to compare DHPF and HPF and to assess their differences.

The present findings showed that IL-1β protein production of PG-treated DHPF and HPF was in a time- and dose-dependent manner. However, IL-1β protein production of
PG-non-treated DHPF during 8 h in the time-course manner was not the same as IL-1β protein production of DHPF concerning 0 μg/ml of PG in the dose-dependent manner. To confirm this result, IL-1β protein production of DHPF and HPF of six other patients when those cells was incubated with 10 μg/ml of L. casei PG for 8 h was examined. As a result, the author considered that IL-1β protein production of PG-non-treated DHPF during 8 h in time-course manner was a reliable value. In addition, from these results of IL-1β protein production of DHPF and HPF of six other patients, IL-1β protein production from PG-treated DHPF and HPF were significantly enhanced compared to IL-1β protein production from PG-non-treated DHPF and HPF. It was also interesting to note in the present study that IL-1β protein production of PG-treated DHPF showed a significant lower level compared with IL-1β protein production of PG-treated HPF. However, IL-1β protein production of PG-non-treated DHPF showed a significant higher level compared with IL-1β protein production of PG-non-treated HPF.

IL-1β has generally been recognized for promoting collagenase production from fibroblasts etc. and playing a tissue-destructive role as a proinflammatory cytokine. Postlethwaite (29) has reported that IL-1 stimulation enhances collagenase production by human foreskin fibroblasts in a dose-dependent manner. Furthermore, Postlethwaite (30) has reported that IL-1β stimulation of a high concentration (27.5 pg/10^5 cells) increases ninefold the rate of collagenase synthesis from human foreskin fibroblasts, compared with IL-1β non-treated human foreskin fibroblasts. In the effect of IL-1β concentration and the proliferation of HPF, Lertchirakarn (35) reported that IL-1β stimulation by concentrations of 10 pg/10^5 cells and above induced dose-dependent inhibition of the proliferation of HPF. Therefore, the author thought that in the present study, IL-1β concentration (17 pg/10^5 cells) of PG-treated DHPF would somewhat inhibit the proliferation of DHPF, and IL-1β concentration (28 pg/10^5 cells) of PG-treated HPF would inhibit the proliferation of HPF more than PG-treated DHPF. In addition, IL-1β concentration (28 pg/10^5 cells) produced from PG-treated HPF in the present study were the same as IL-1β concentration (27.5 pg/10^5 cells) of Postlethwaite’s study. (30) From this fact of the similar IL-1β concentration (28 pg/10^5 cells), the author thought that collagenase synthesis induced from PG-treated HPF in this study would enhance as well as the collagenase synthesis in Postlethwaite’s study. (30) IL-1β production of PG-treated DHPF was less than PG-treated HPF; as a result, collagenase synthesis produced from PG-treated DHPF would also be less than collagenase synthesis produced from PG-treated HPF. Hence, PG-treated DHPF would have less tissue...
destruction than PG-treated HPF.

In regard to IL-1β protein production of PG-non-treated DHPF showing a significant higher level compared with IL-1β protein production of PG-non-treated HPF. Uitto reported on PPH activity collagen synthesis in the pulp of healthy human teeth. In the pulp of human deciduous teeth, PPH activity presents about one and a half-fold enhancement, compared with the pulp of human permanent teeth. In the effect of IL-1β and collagen synthesis from HPF of healthy dental pulp, Barkhordar reported that IL-1β stimulation of a low concentration (5–10 pg/10^5 cells) significantly increases about twofold collagen synthesis from HPF of healthy dental pulp compared with IL-1β of non-treated HPF of healthy dental pulp.

From these reports, the author thought that PPH activity of PG-non-treated DHPF would be higher than PPH activity of PG-non-treated HPF; as a result, PG-non-treated DHPF would present collagen production more than PG-non-treated HPF. Therefore, for an increase in the collagen production, IL-1β production of PG-non-treated DHPF would have also increased more than IL-1β production of PG-non-treated HPF.

In summary, with L. casei PG stimulation, the pulp of the deciduous teeth will have less collagenase synthesis from dental-pulp-derived fibroblasts than the pulp of permanent teeth; as a result, the pulp of the deciduous teeth will have less tissue destruction than the pulp of permanent teeth. This supports the belief of pediatric dentists that there is a lower sensitivity to pain in deciduous teeth than in permanent teeth. (31) Without L. casei PG stimulation, the pulp of the deciduous teeth will have increased collagen synthesis from dental-pulp-derived fibroblasts in comparison to the pulp of permanent teeth, so that the pulp of the deciduous teeth will have proliferation ability of dental-pulp-derived fibroblasts higher than the pulp of permanent teeth. This will also support the idea that the human pulp-derived fibroblasts from the younger donors have a more higher survival rates in primary cultures than the human pulp derived fibroblasts from the older donors. (36)

To identify IL-1β protein production through the elevation of mRNA expression, the author examined RT-PCR analysis in DHPF and HPF. The results showed that IL-1β mRNA levels of PG-treated DHPF and HPF were clearly enhanced, compared to IL-1β mRNA levels of PG-non-treated DHPF and HPF. IL-1β mRNA level of PG-treated DHPF showed a lower level, compared to IL-1β mRNA level of PG-treated HPF. These results in IL-1β mRNA levels of DHPF and HPF had been enhanced to similar relations as IL-1β protein production. IL-1β protein production of PG-treated DHPF and HPF were clearly enhanced, compared to IL-1β protein production of PG-non-treated DHPF and HPF. IL-1β protein production of PG-treated DHPF showed a lower level, compared to IL-1β protein production of PG-treated HPF. These present findings indicate that IL-1β protein production from DHPF and HPF stimulated by L. casei PG had increased through the enhancement of IL-1β mRNA.

These findings suggest that pulpitis in deciduous teeth would be induced less than pulpitis in permanent teeth because both IL-1β protein production and IL-1β mRNA expressions of deciduous teeth were clearly lower compared with IL-1β protein production and IL-1β mRNA expressions of permanent teeth. L. casei PG would also stimulate IL-1β production through enhancement of IL-1β mRNA expression from DHPF and HPF because it clearly enhanced both IL-1β protein production and IL-1β mRNA expressions from DHPF and HPF.

Further research is needed to confirm the various MMPs activity and collagen synthesis of DHPF and HPF.

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Bibliography

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Reference


