ORIGINAL ARTICLE

γ-D-glutamyl-meso-diaminopimelic Acid (iE-DAP) Regulates the Production of Matrix Metalloproteinase-1 by Human Dental Pulp Fibroblast-like Cells

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Synopsis

Nucleotide-binding oligomerization domain 1, which is constitutively expressed in human dental pulp fibroblast-like cells (hDPFs), plays a role in pulpal immune responses by sensing γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) from bacteria. Matrix metalloproteinase-1 (MMP-1) is an important enzyme that degrades type I collagen, an extracellular matrix component, and is involved in the destruction of dental pulp tissue. In the present study, we investigated the involvement of the ERK1/2 signaling pathway in MMP-1 production by iE-DAP-stimulated hDPFs. iE-DAP was not cytotoxic against hDPFs and did not affect their proliferation. A stimulation with iE-DAP enhanced MMP-1 production and ERK1/2 phosphorylation. The ERK1/2 signaling pathway was involved in the production of MMP-1 by iE-DAP-stimulated hDPFs. These results suggest that the induction of MMP-1 production by iE-DAP-stimulated hDPFs exacerbates pulpitis by promoting the degradation of surrounding collagen.

***Key words**: iE-DAP, MMP-1, ERK1/2, human dental pulp fibroblast-like cells*

Introduction

Matrix metalloproteinases (MMPs) are involved in a wide range of pathological and physiological processes, including wound healing, morphogenesis and inflammation. Inflammatory pulp destruction, which occurs in pulpitis, is partially controlled by MMPs and tissue inhibitors of metalloproteinases [1]. The extracellular matrix (ECM), which constitutes the connective tissue of the pulp, is degraded by MMPs with pulpitis. MMPs have been classified into collagenases (MMP-1 and MMP-8), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11) and membrane-type MMPs (MT-MMPs) [2,3]. MMP-1, MMP-2, MMP-3, and MT1-MMP levels were previously shown to be significantly higher in inflamed pulpal and periapical tissues than in healthy tissues [4]. MMPs are secreted from pulp tissue and play important roles in the formation of the dentin matrix, the regulation of caries progression, and secondary dentin formation. Previous findings support the fundamental role of MMPs in oral tissue remodeling and destruction [1]. MMP-1 is a major proteolytic enzyme that cleaves type 1 collagen and may be a key enzyme in the destruction of connective tissue [5]. The innate immune system is the first line of defense and plays an important role in the pathogenesis of many inflammatory diseases [6].

The detection of diverse pathogen-associated molecular patterns (PAMPs)

post-infection by specific pattern recognition receptors (PRRs) is important for alerting the body to the presence of potential harmful pathogens. The PRR family that detects PAMPs consists of membrane-bound receptors, such as Toll-like receptors, and cytoplasmic receptors, including nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [7,8]. Many inflammatory diseases are associated with NLRs, and recent studies emphasized the immunological importance of these receptors [9]. NOD1 is a member of NLRs and responds to γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP). iE-DAP is primarily present in the peptidoglycan of Gram-negative bacteria and some Gram-positive bacteria, such as *Bacillus* spp. and *Listeria* spp. [10,11]. iE-DAP is recognized by NOD1, a cytosolic PRR, as PAMP and generates proinflammatory immune responses [11]. Since NOD1 is expressed in healthy human dental pulp fibroblasts [12], these cells appear to play a role in proinflammatory immune responses against cariogenic bacteria. The binding of iE-DAP to NOD1 activates MAP kinase (p38, ERK1/2, and JNK) pathways, which induce innate immune responses [13].

We previously reported that iE-DAP promoted the production of MMP-3 by human dental pulp cells [14]. However, the relationship between MMP-1 production and the iE-DAP/NOD1 signaling pathway, including MAP kinases, in hDPFs remains unclear. Therefore, the present study investigated the iE-DAP/NOD1-induced production of MMP-1 and the role of its signaling cascade in hDPFs.

Before confirming the effect of iE-DAP on MMP-1 production, we investigated that iE-DAP does not affect cell proliferation or cell death by flow cytometry and Cell Counting Kit-8. To confirm the effect of iE-DAP on MMP-1 production, Western blotting was performed to visually distinguish between pro-MMP and activated MMP. In addition, we examined the phosphorylation of MAP kinase using Western blotting to investigate the intracellular signaling pathway in MMP-1 production.

Materials and Methods

1. Cell culture

hDPFs were obtained from the non-carious pulp tissue of healthy patients (any age, gender and tooth type) undergoing treatment at Osaka Dental University Hospital. The collected pulp tissue was placed into a 25 mL cell culture flask (Corning, NY, USA), cut at small pieces with the aid of 15c scalpel blade, and then, immersed in the supplemented medium. hDPFs were obtained from the non-carious pulp tissue of healthy patients undergoing treatment at Osaka Dental University Hospital. Primary cultures were grown in α-modified Minimum Essential Medium (α-MEM; FUJIFILM Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Cosmo Bio, Tokyo, Japan), 2 mM L-glutamine (FUJIFILM Wako Pure Chemical), 100 μg/mL penicillin (FUJIFILM Wako Pure Chemical), and 100 μg/mL streptomycin (FUJIFILM Wako Pure Chemical) at 37°C in an atmosphere of 5% CO_2-95% air. The first subcultures were obtained 20 to 30 days later, maintained at 37°C in an atmosphere of 5% CO_2-95% air, and routinely subcultured after the addition of trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA·4Na, (Nacalai Tesque, Kyoto, Japan)) for cell release. Experiments with hDPFs were performed between passages 3 and 10. The present study was approved by the Ethical Review Board of Osaka Dental University (Approval No.111112). Informed consent was obtained from all participants and the study was conducted according to the principles of the Declaration of Helsinki.

2. Cytotoxic effects of iE-DAP on hDPFs

hDPFs (1×10^5 cells/well) were incubated in a 12-well plate at 37°C for 24 hours in the presence of 5% CO_2. After the addition of 10 μg/mL iE-DAP (InvivoGen, San Diego, CA, USA) and an incubation for an additional 24 hours, hDPFs were collected. Negative control cells were treated with 0.5% saponin for 10 min. 7-AAD (5 μg/mL, BD Bioscience) was added to remove dead cells. Cells were washed with FACS buffer (PBS (-) containing 1% FBS and 0.1% sodium azide (Wako Pure Chemical)), and 10,000 stained cells per assay were evaluated using BD FACSVerse (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed by FlowJO software (BD Biosciences).

3. Effects of iE-DAP on the proliferation of hDPFs

hDPFs were seeded on a 96-well plate at a density of 1×10^5 cells/well, and incubated with or

without 10 μg/mL iE-DAP at 37°C for 24 hours in the presence of 5% CO_2. Cell proliferation was assessed by measuring absorbance (450 nm) on SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA) using Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan). Experiments were performed independently 4 times. All values are expressed as means ± standard deviations. Statistical analyses were conducted using the Student's *t*-test, with a P value <0.05 considered to be significant. Each sample was tested in triplicate.

4. Western blotting

hDPFs were seeded on a 12-well plate at a density of 1.0×10^5 cells/well and cultured for 2 days to 90% confluency in α-MEM containing 10% FBS. hDPFs were incubated in serum-free α-MEM for 24 hours. After the incubation,

hDPFs were stimulated with iE-DAP (0, 1, 5, 10, and 20 μg/mL) for 24 hours. Conditioned medium was collected, centrifuged to remove debris, concentrated up to 30-fold with Amicon Ultra (Merck KGaA, Darmstadt, Germany), and proteins were visualized by Western blotting. Whole-cell lysates were prepared by lysing cells with sample buffer (containing 0.25 M Tris HCl pH 6.8, 8% SDS, 40% glycerol, 0.02% bromophenol blue, and 4% 2-mercaptoethanol). Equal amounts of each sample were then separated on 10% SDS/PAGE. Following their separation, proteins were electrophoretically transferred to polyvinylidene fluoride membranes (Merck KGaA) and blocked with 10% Blocking One (Nacalai) in 50 mM Tris–HCl at pH 7.5 and 150 mM NaCl overnight. Membranes were treated with MMP-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), used at a 1:1000 dilution, and also with β-actin antibodies (Santa Cruz Biotechnology), used at a 1:1000 dilution, at room temperature for 1 hour. A horseradish peroxidase (HRP)-conjugated secondary antibody, mouse anti-goat IgG-HRP (Santa Cruz Biotechnology), was used at a 1:2000 dilution and the chemiluminescent reagent, Immobilon Western Chemiluminescent HRP Substrate (Merck KGaA) was employed to visualize immunoreactive bands. Blot images were acquired using ChemiDoc MP (Bio-Rad, Hercules, CA, USA).

In an experiment on ERK1/2 phosphorylation, hDPFs were pretreated in the same manner. hDPFs were stimulated with 10 μg/mL iE-DAP for 10 minutes. After the stimulation, total protein was isolated in sample buffer supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Western blotting was similarly performed using an anti-phospho ERK1/2 antibody (Cell Signaling Technology, Danvers, MA, USA) at 1000:1. Membranes were treated with secondary antibodies in the same manner and visualized phosphorylated bands were obtained using the ChemiDoc MP. The same membrane was stripped and reprobed with an anti-ERK1/2 antibody (Cell Signaling Technology) at 1:1000. Blot images were acquired using ChemiDoc MP.

Results

1. Cytotoxic effects of iE-DAP on hDPFs.

The cytotoxicity of hDPFs was examined by flow cytometry.—Compared to unstimulated control cells, saponin-treated negative control dead cells were positive for 7-AAD, and shifted to the right on histograms. iE-DAP stimulated cells were negative for 7-AAD. Histograms of unstimulated and iE-DAP stimulated cells were almost identical, and no rightward shift of iE-DAP stimulated cells was observed compared to unstimulated cells. The results obtained revealed that iE-DAP was not cytotoxic against hDPFs at the concentrations used in this experiment (Fig. 1).

2. Effects of iE-DAP on the proliferation of hDPFs.

The proliferation of hDPFs was examined using Cell Counting Kit-8. The proliferation of cells stimulated with 10 μg/mL iE-DAP showed no significant difference from that of control cells (Fig. 2). These results revealed that iE-DAP did not affect the proliferation of hDPFs at the concentrations used in this experiment.

3. iE-DAP enhanced the production of MMP-1 on hDPFs

The dose-dependent production of MMP-1 was observed in hDPFs cultured in the presence of 1, 5, 10, and 20 μg/mL iE-DAP, with a peak at 10 μg/mL iE-DAP (Fig. 3). Neither the total amount of β-actin nor the activation status was affected by the stimulation with iE-DAP. These results indicated that iE-DAP specifically stimulated the production of MMP-1 by hDPFs.

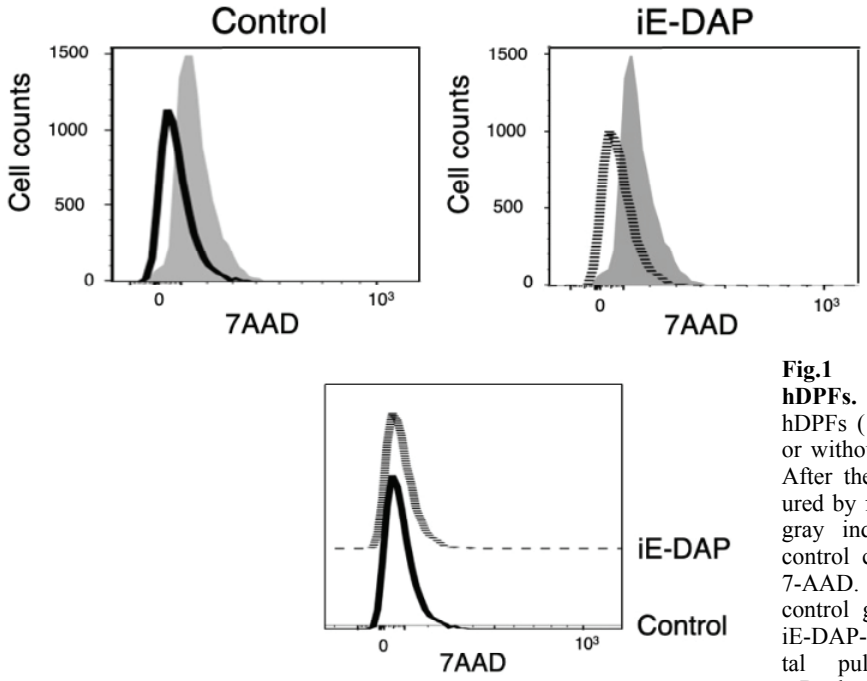

Fig.1 Cytotoxic effects of iE-DAP on hDPFs.
hDPFs (1×10^5 cells/well) were incubated with or without 10 μg/mL of iE-DAP for 24 hours. After the incubation, cell viability was measured by flow cytometry. In the top two graphs, gray indicates that saponin-treated negative control cells are dead cells and stained with 7-AAD. The solid line shows the unstimulated control group and the dotted line shows the iE-DAP-stimulated group. hDPFs, human dental pulp fibroblast-like cells; iE-DAP, γ-D-glutamyl-meso-diaminopimelic acid

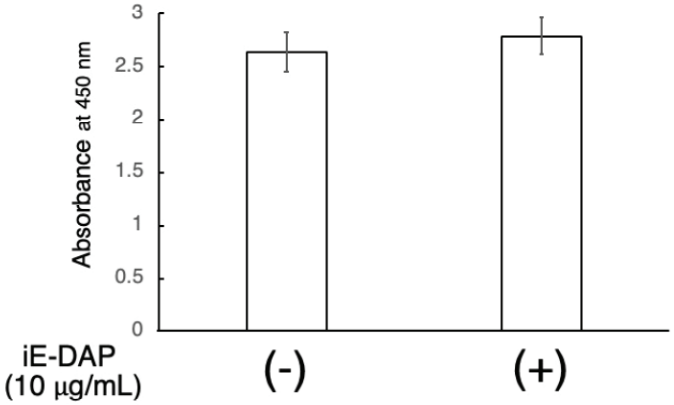

Fig.2 Proliferation effects of iE-DAP on hDPFs.
hDPFs (1×10^4 cells/well) were seeded on a 96-well plate and incubated with or without 10 μg/mL iE-DAP at 37°C for 24 hours. The proliferation of hDPFs was examined using Cell Counting Kit-8. Cell proliferation was assessed by measuring absorbance (450 nm). Each sample was tested in triplicate. All values are expressed as means ± standard deviations. Experiments were performed independently 4 times. hDPFs; human dental pulp fibroblast-like cells; iE-DAP; γ-D-glutamyl-meso-diaminopimelic acid

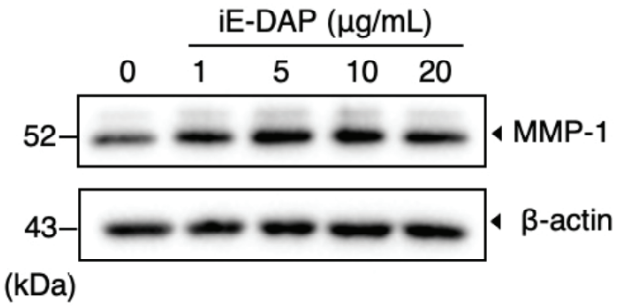

Fig. 3 Effects of iE-DAP on MMP-1 production by hDPFs
hDPFs (1×10^5 cells/well) were incubated in a 12-well plate at 37°C for 24 hours. Cells were then incubated in serum-free α-MEM for 24 hours. After the incubation, hDPFs were cultured in serum-free α-MEM containing iE-DAP (0, 1, 5, 10, and 20 μg/mL) for 24 hours. Western blotting was performed to evaluate MMP-1 production. Actin was used as the loading control. The 52-kDa band is MMP-1. The 43-kDa band is β-actin. hDPFs, human dental pulp fibroblast-like cells; α-MEM, alpha-modified Minimum Essential Medium; iE-DAP; γ-D-glutamyl-meso-diaminopimelic acid; MMP-1, matrix metalloproteinase-1

4. iE-DAP enhanced ERK1/2 phosphorylation on hDPF cells.

ERK1/2 was constitutively phosphorylated without the stimulation, and the iE-DAP stimulation enhanced ERK1/2 phosphorylation slightly more than in unstimulated hDPFs (Fig.4, upper panel). To ensure that equal amounts of ERK1/2 were obtained from the lysates, membranes were stripped and reprobed with the anti-ERK1/2 antibody. The results obtained revealed that equal amounts of ERK1/2 were produced by lysates obtained from each sample (Fig. 4, lower panel).

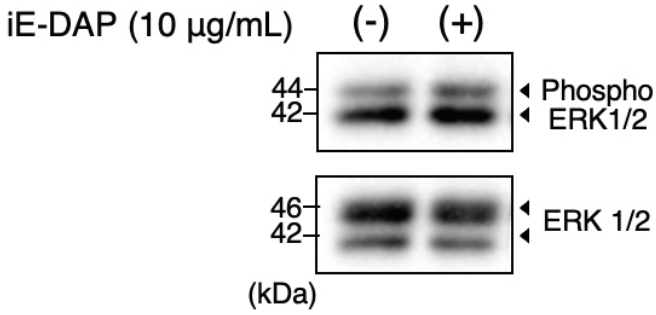

Fig 4 Effects of iE-DAP on ERK1/2 phosphorylation in hDPFs

hDPFs were stimulated with 10 μg/mL iE-DAP for 10 min. Cell lysate samples were prepared. The phosphorylation level of ERK1/2 was examined by Western blotting (upper). To ensure that equal amounts of ERK1/2 were obtained from lysates, membranes were stripped and reprobed with anti- ERK1/2 antibodies (lower). Four samples were analyzed and a typical image is shown for each. hDPF, human dental pulp fibroblast-like cells; iE-DAP; γ-D-glutamyl-meso-diaminopimelic acid

Discussion

The present results revealed that the activation of iE-DAP/NOD1 in hDPFs induced the production of MMP-1 via the ERK1/2 phosphorylation of MAP kinase signaling. The expression of NOD1 has been detected in human gingival fibroblasts and human dental pulp fibroblasts [15, 16]. These findings showed that resident tissue cells expressed innate immune receptors and played an active role in the recognition of and responses to invading pathogens. Specific bacterial components activate innate immune receptors, which induce an inflammatory reaction. As a result of inflammation, endogenous ligands, danger (or damage)-associated molecular patterns, are released from tissue and stimulate innate immune reactions in a positive feedback mechanism. A recent study showed that NOD1 and NOD2 were constitutively expressed in human pulp fibroblasts and were involved in the pulp immune response that induced pulpitis [17]. Previous studies reported that several MMPs were expressed in pulpitic tissue and played a role in pulp tissue destruction [18]. MMP-1 degrades type 1 collagen and is involved in the destruction of periodontal soft tissue in periodontal lesions [19,20]. TNF-α-stimulated enhancements in MMP-1 in dental pulp cells were found to contribute to the development of pulpitis [21]. Pulpitis develops through the stimulation of NOD1 and inflammatory cytokine expression [22]. Since the production of MMP-1 plays an important role in pulpitis, we examined the signaling cascade that produces MMP-1 in iE-DAP-stimulated hDPFs. The results obtained clearly showed that the activation of iE-DAP/NOD1 increased the production of MMP-1. A previous study demonstrated that iE-DAP/NOD1 activated MAP kinases [23]. We also found that the activation of JNK played a key role in the induction of MMP-3 from iE-DAP stimulated-hDPFs [14]. However, the mechanisms underlying the production of MMP-1 from iE-DAP stimulated-hDPFs have not yet been elucidated. Regarding the production of MMP-1 by iE-DAP/NOD1-stimulated hDPFs, the present study showed that the iE-DAP/NOD1 stimulation activated MAP kinases, particularly ERK1/2.

Conclusion

The present study demonstrated that iE-DAP induced the production of MMP-1 via ERK1/2-activating signaling pathways in hDPFs. The results obtained suggest that the induction of MMP-1 production exacerbates pulpitis by promoting the degradation of surrounding collagen. Since MMPs are involved in ECM metabolism and form a complex network, further studies on the regulatory mechanisms of other MMPs in the development of pulpitis are warranted. The results of this research may lead to the development of new therapeutic drugs using nano drug delivery systems in the future.

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Conflict of Interest
The authors declare that they have no competing interests.

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