ORIGINAL ARTICLE

Muramyl Dipeptide (MDP) Regulates the Production of Matrix Metalloproteinase-3 by Human Dental Pulp Fibroblast-like Cells

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Synopsis

Nucleotide-binding oligomerization domain 2, which is constitutively expressed in human dental pulp fibroblast-like cells (hDPFs), plays a role in pulpal immune responses by sensing muramyl dipeptide (MDP) from bacteria. Matrix metalloproteinase-3 (MMP-3) is abundantly expressed in acute pulpitis and acts as a mediator of the healing of dental pulp as an anti-inflammatory and regenerative factor. In the present study, we investigated the involvement of the ERK1/2 signaling pathway in MMP-3 production by MDP-stimulated hDPFs. MDP was not cytotoxic against hDPFs and did not affect their proliferation. A stimulation with MDP enhanced MMP-3 production and ERK1/2 phosphorylation. The ERK1/2 signaling pathway was involved in the production of MMP-3 by MDP-stimulated hDPFs. These results suggest that the induction of MMP-3 production by MDP-stimulated hDPFs has an anti-inflammatory function by degrading inflammatory cytokines.

***Key words**: MDP, MMP-3, ERK1/2, human dental pulp fibroblast-like cells*

Introduction

In teeth with deep caries and exposed pulp, bacteria directly invade the dental pulp, which leads to irreversible pulpitis. The innate immune response is the first line of defense and plays an important role in regulating caries-induced pulpal inflammation [1]. Dental pulp cells, which are the main constituents of dental pulp tissue, express pattern recognition receptors (PRRs). The recognition of microbe-specific molecules, called pathogen-associated molecular patterns (PAMPs), by PRRs stimulates the innate immune response, thereby leading to the activation of inflammation [2-4]. The PRR family comprises membrane-bound receptors, such as Toll-like receptors, and cytoplasmic receptors, including nucleotide-binding oligomerization domain-like and leucine-rich repeat receptors (NLRs). The first NLRs to be identified were nucleotide-binding oligomerization domain 1 (NOD1) and NOD2, which recognize peptidoglycan components common to both Gram-positive and -negative bacteria. They promote the activation of the mitogen-activated protein (MAP) kinases and nuclear factor kappa-b (NF-κB) pathways, resulting in the production of inflammatory cytokines [5]. Muramyl dipeptide (MDP) is a constituent of both Gram-positive and -negative bacteria and is recognized as a PAMP by NOD2 to generate proinflammatory immune responses [6]. Since NOD2 is expressed in healthy human dental

pulp fibroblasts [7], cariogenic bacteria appear to stimulate the innate immune response of human dental pulp fibroblasts, which leads to the activation of inflammation.

Matrix metalloproteinases (MMPs) have been implicated in the degradation of extracellular matrix (ECM) components, which regulate various physiological and pathological processes [8]. MMPs play an important role in wound healing by modifying the wound matrix, thereby enabling tissue remodeling [4]. They also have a dual role in the pathogenesis of inflammation by causing tissue destruction and stimulating a protective immune response [9]. MMPs are classified into collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10 and MMP-11), matrilysins (MMP-7 and MMP-26), and membrane-type MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25) [10,11]. MMP-3 plays an essential role in pulpitis. Shin *et al*. report that MMP3 concentrations are higher in acute pulpitis than in normal pulp tissue [12]. Previous studies have demonstrated the function of MMP-3 as a mediator in the healing of dental pulp, acting as an anti-inflammatory factor. These studies have also shown that MMP-3 promotes fibroblast wound healing and assists in angiogenesis in pulpitis [13].

The binding of MDP to NOD2 activates the MAP kinases and NF-κB pathways, resulting in the production of inflammatory cytokines [5,14]. We previously reported that iE-DAP promoted the production of MMP-3 by human dental pulp cells [15]. However, the relationship between MMP-3 production and the MDP/NOD2 signaling pathway, including MAP kinases, in hDPFs remains unclear. Therefore, the present study investigated the MDP/NOD2-induced production of MMP-3 and the role of its signaling cascade in hDPFs.

Materials and Methods

1. Cell culture

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. Cytotoxicity experiment

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 MDP (InvivoGen, San Diego, CA, USA) and further incubation for 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. Cell proliferation experiment

hDPFs were seeded on a 96-well plate at a density of 1×10^4 cells/well and then incubated with or without 10 μg/mL MDP 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 MDP (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-3 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 p38 and ERK1/2 phosphorylation, hDPFs were pretreated in the same manner. hDPFs were stimulated with 10 μg/mL MDP 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) or anti-phospho p38 antibody (Santa Cruz Biotechnology) at 1000:1. Membranes were similarly treated with secondary antibodies and visualized phosphorylated bands were obtained using ChemiDoc MP. The same membrane was stripped and reprobed with an anti-ERK1/2 antibody (Cell Signaling Technology) or anti-p38 antibody (Santa Cruz Biotechnology) at 1:1000. Blot images were acquired using ChemiDoc MP.

Results

1. Cytotoxic effects of MDP on hDPFs.

We initially examined the effects of MDP on cytotoxicity. hDPFs were incubated in a 12-well plate (1×10^5 cells/well) with 10 μg/mL of MDP for 24 hours. 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. MDP stimulated cells were negative for 7-AAD. Histograms of unstimulated and MDP stimulated cells were almost identical, and no rightward shift of MDP stimulated cells was observed compared to unstimulated cells. The results obtained revealed that MDP was not cytotoxic against hDPFs at the concentrations used in this experiment (Fig. 1).

2. Effects of MDP on the proliferation of hDPFs.

We examined the effects of MDP on cell proliferation. hDPFs were seeded on a 96-well plate and incubated with or without 10 μg/mL MDP at 37°C for 24 hours. The proliferation of hDPFs was examined using Cell Counting Kit-8. The proliferation of cells stimulated with 10 μg/mL MDP showed no significant difference from that of control cells (Fig. 2). The results obtained revealed that MDP did not affect the proliferation of hDPFs at the concentrations used in this experiment.

3. MDP enhanced the production of MMP-3 by hDPFs

We previously reported that TNF-α-stimulated hDPFs produced MMP-3 [16]. Therefore, we herein investigated whether MDP affected the production of MMP-3 by hDPFs. The dose-dependent production of MMP-3 was observed in hDPFs cultured in the presence of 1, 5, 10, and 20 μg/mL MDP, with a peak at 10 μg/mL MDP (Fig. 3). Neither the total amount of β-actin nor the activation status was affected by the stimulation with MDP. These results indicated that MDP specifically stimulated the production of MMP-3 in hDPFs.

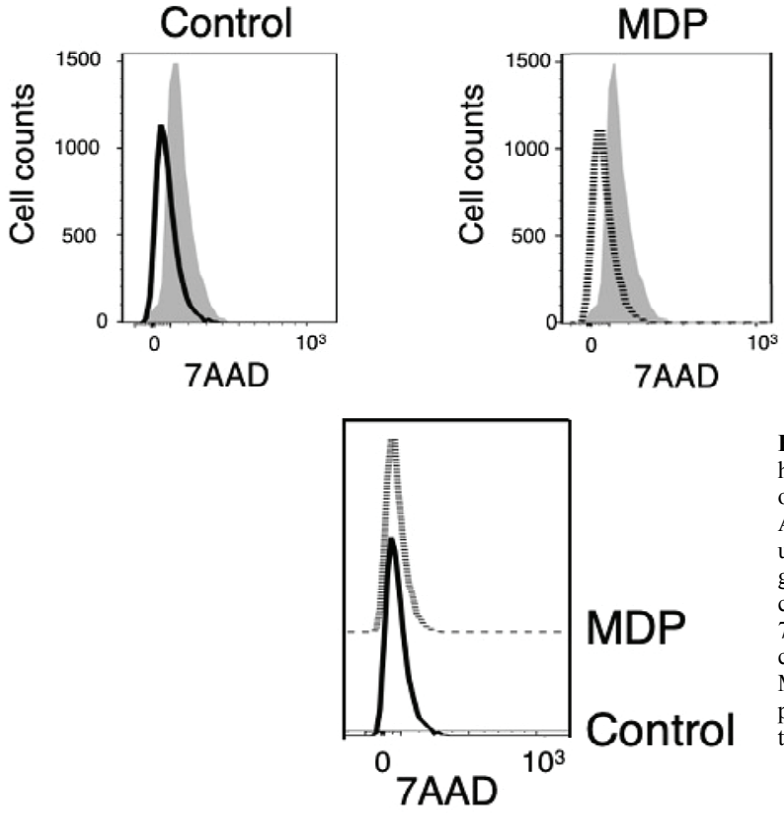

Fig.1 Cytotoxic effects of MDP on hDPFs.
hDPFs (1×10^5 cells/well) were incubated with or without 10 μg/mL of MDP for 24 hours. After the incubation, cell viability was measured by flow cytometry. In the top two graphs, gray indicates that saponin-treated negative control cells were dead cells and stained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

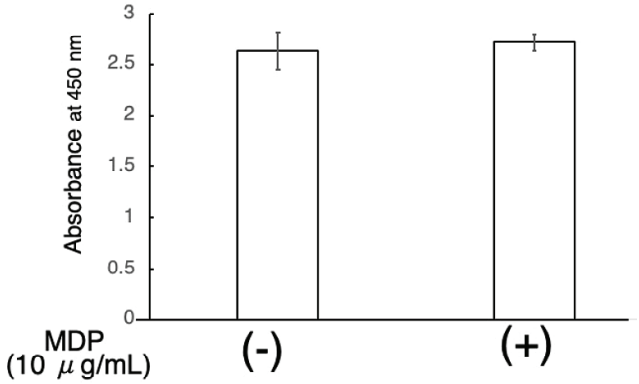

Fig.2 Effects of MDP on the proliferation of hDPFs.
hDPFs (1×10^4 cells/well) were seeded on a 96-well plate and incubated with or without 10 μg/mL MDP at 37°C for 24 hours. The proliferation of hDPFs was examined using Cell Counting Kit-8. Cell proliferation was assessed by measuring absorbance (450 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; MDP, muramyl dipeptide

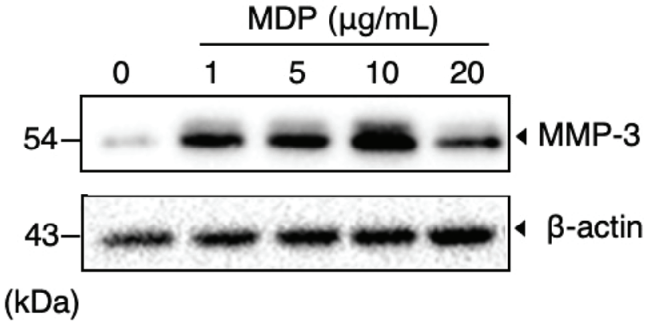

Fig. 3 Effects of MDP on MMP-3 production in hDPFs
hDPFs (1×10^5 cells/well) were incubated in a 12-well plate at 37°C for 24 hours. Cells were then incubated in serum-free a-MEM for 24 hours. After the incubation, hDPFs were cultured in serum-free a-MEM containing MDP (0, 1, 5, 10, and 20 μg/mL) for 24 hours. Western blotting was performed to assess MMP-3 production. Actin was used as the loading control. The 54-kDa band is MMP-3. The 43-kDa band is β-actin. hDPFs, human dental pulp fibroblast-like cells; α-MEM, alpha-modified Minimum Essential Medium; MDP, muramyl dipeptide; MMP-3, matrix metalloproteinase-3

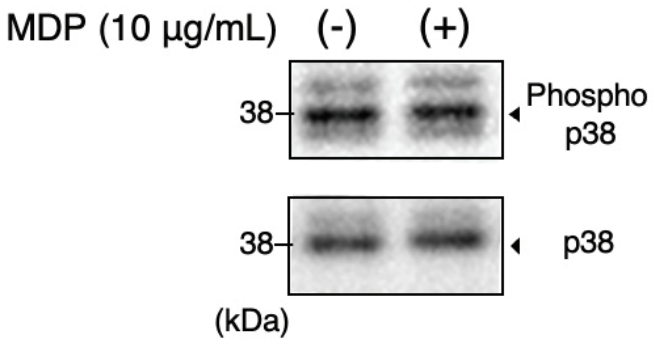

Fig 4 Effects of MDP on p38 phosphorylation in hDPFs
hDPFs were stimulated with 10 μg/mL MDP for 10 min. Cell lysate samples were prepared. The phosphorylation level of p38 was examined by Western blotting (upper). To ensure that equal amounts of p38 were obtained from lysates, membranes were stripped and reprobed with anti-p38 antibodies (lower). Four samples were analyzed and a typical image is shown for each. hDPF, human dental pulp fibroblast-like cells. MDP, muramyl dipeptide.

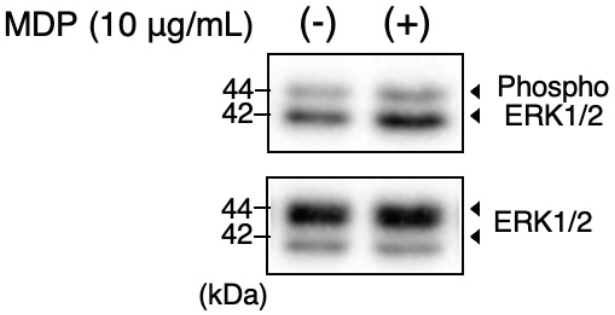

Fig 5 Effects of MDP on ERK1/2 phosphorylation in hDPFs
hDPFs were stimulated with 10 μg/mL MDP for 10 min. Cell lysate samples were prepared. The phosphorylation level of ERK1/2 was examined by Western blotting (upper). To ensure that equal amounts of ERK1/2 were obtained from lysates, membranes were 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; MDP, muramyl dipeptide.

4. Effects of MDP on the activation of MAP kinases in hDPF cells.

A previous study reported that MDP activated MAP kinases [14]. The role of p38 and ERK1/2 in MMP-3 production by MDP was examined herein using Western blotting. We investigated the phosphorylation of p38 and ERK1/2 in hDPFs stimulated with 10 μg/mL MDP for 10 min. p38 was constitutively phosphorylated even without the stimulation. No change was observed in the phosphorylation of p38 after the MDP stimulation (Fig.4, upper panel). To ensure that equal amounts of p38 were obtained from the lysates, membranes were stripped and reprobed with the anti-p38 antibody. The results obtained revealed that equal amounts of p38 were created by the lysates obtained from each sample (Fig. 4, lower panel). ERK1/2 was constitutively phosphorylated without the stimulation, and the MDP stimulation enhanced ERK1/2 phosphorylation slightly more than in unstimulated hDPFs (Fig.5, upper panel). Using a similar method for p38, we revealed that equal amounts of ERK1/2 were created by the lysates obtained from each sample (Fig. 5, lower panel).

Discussion

The present results revealed that the activation of MDP/NOD2 in hDPFs induced the production of MMP-3 via the ERK1/2 phosphorylation of MAP kinase signaling. The expression of NOD2 has been detected in human gingival fibroblasts and human dental pulp fibroblasts [5,17]. These findings showed that dental pulp tissue cells express innate immune receptors and play an important role in recognizing and responding to invading pathogens. The first step of innate immune detection is PRRs recognizing microbe-specific molecules, known as PAMPs. In dental pulp tissue, PRRs expressed by hDPFs activate the innate immune response by recognizing PAMPs. A recent study showed that NOD1 and NOD2 were constitutively expressed in human pulp fibroblasts and were involved in the pulp immune response to induce pulpitis [5].

MMP-3 exhibits broad substrate specificity to ECM [18] and acts as a mediator in the healing of dental pulp as an anti-inflammatory and regenerative factor [19]. MMP-3 has been reported to directly degrade active cytokines, such as IL-1β, 19, and 20 and chemokines [20],

and is considered to have an anti-inflammatory function [21]. MMP-3 concentrations are known to be higher in acute pulpitic tissue than in normal pulp tissue [12]. Since the production of MMP-3 plays an important role in pulpitis, we examined the signaling cascade that produces MMP-3 in MDP-stimulated hDPFs. The results obtained clearly showed that the activation of MDP/NOD2 increased the production of MMP-3. The activation of MAP kinases via MDP/NOD2 has been reported in murine bone marrow–derived macrophages, in which p38, ERK1/2, and JNK were phosphorylated after a stimulation with MDP [22]. Regarding the MDP/NOD2 signaling pathway in hDPFs, we herein showed that the MDP stimulation activated ERK1/2, but not p38, among MAP kinases. However, the mechanisms underlying the production of MMP-3 by MDP-stimulated hDPFs have not yet been elucidated.

Conclusion

The present study suggests that MDP induced the production of MMP-3 via ERK1/2-activating signaling pathways in hDPFs. The results obtained indicate that the innate immune response of dental pulp cells was activated by the NOD2 stimulation to produce MMP3, which promoted anti-inflammatory effects. Since MMPs form a complex network, the regulatory mechanisms of other MMPs in the immune response of dental pulp cells warrant further study.

Acknowledgments

We are grateful to Dr. Mao from the Graduate chool of Dentistry, the Department of Physiology, Osaka Dental University.

This work was supported by the Japan Society for the Promotion of Science KAKENHI Grant Number JP21K10269 (Grant-in-Aid for Scientific Research (C)).

Conflict of Interest

The authors declare that they have no competing interests.

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(Received: May 28, 2023/
Accepted: Jun 20, 2023)

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