Dental Materials Journal 2023; 42(3): 375–382

Effects of a co-stimulation with S-PRG filler eluate and muramyl dipeptide (MDP) on matrix metalloproteinase-1 production by human dental pulp fibroblast-like cells

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The present study investigated the effects of a co-stimulation with surface reaction-type pre-reacted glass-ionomer (S-PRG) filler eluate and muramyl dipeptide (MDP) on matrix metalloproteinase (MMP)-1 production by human dental pulp fibroblast-like cells (hDPFs). S-PRG filler eluate contains 6 ions (F, Na, Al, B, Sr, and Si) released from S-PRG filler. Each S-PRG filler eluate and MDP stimulation enhanced MMP-1 production by hDPFs. The co-stimulation with S-PRG filler eluate and MDP enhanced MMP-1 production more than the MDP stimulation alone. A similar stimulation induced the phosphorylation of ERK 1/2. The increased secretion of MMP-1 and enhanced phosphorylation of ERK 1/2 by the co-stimulation with S-PRG filler eluate and MDP were suppressed by the selective and potent CaSR antagonist NPS 2143. Since strontium binds to CaSR, these results suggest that the enhanced production of MMP-1 by the co-stimulation with S-PRG filler eluate and MDP was due to the effects of strontium.

Keywords: S-PRG filler eluate, Muramyl dipeptide, Human dental pulp fibroblast-like cells, Matrix metalloproteinase

INTRODUCTION

Inflammatory pulp destruction, which occurs in reversible and irreversible pulpitis, is partially controlled by matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs[1)]. The connective tissue of pulp is composed of an extracellular matrix (ECM) that is degraded by MMPs with pulpitis. MMP family proteins play dual roles in the pathogenesis of inflammation, stimulating protective innate and/or adaptive immune functions as well as tissue destruction. MMPs have been classified into MMP-1 and -8 (tissue collagenase), MMP-2 and -9 (gelatinases A and B), MMP-3, -10, and -11 (stromelysins-1, -2, and -3), MMP-7, membrane-type MMPs (MT-MMPs), and other 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 have been isolated from dentine, pulp tissue, and odontoblasts, where they play an important role in the formation of the dentine matrix, modulating the progression of caries and the secondary formation of dentine[4)]. Previous findings support the fundamental role of MMPs during the development, remodeling, and destruction of oral tissues[1)]. Since MMP-1 is a collagenase, it degrades type 1 collagen in pulpitis.

The activation of the innate immune system is characterized by the detection of pathogens *via* pattern recognition receptors (PRRs), which trigger an inflammatory response. PRRs recognize microbe-specific molecules called pathogen-associated molecular patterns (PAMPs). PAMP-recognized PRRs alert the human body to the presence of potential harmful pathogens and activate the innate immune system. The PRR family comprises membrane-bound receptors, such as Toll-like receptors (TLRs), and cytoplasmic receptors, including nucleotide-binding oligomerization domain–like and leucine-rich repeat receptors (NLRs). NLRs share similarities with TLRs in that their leucine-rich repeat (LRR) domain mediates ligand binding. There are 2 major subfamilies of NLRs: nucleotide-binding oligomerization domains (NODs) and NACHT, LRR, and pyrin domains. NOD2 is an intracellular Nod protein that contains a nucleotide-binding site domain flanked by a LRR[5)]. Muramyl dipeptide (MDP), a constituent of both gram-positive and -negative bacteria, is recognized by NOD2, a cytosolic PRR, as PAMP and generates proinflammatory immune responses[6)]. Since NOD2 is expressed in healthy human dental pulp fibroblasts[7)], human dental pulp fibroblasts appear to play a role in proinflammatory immune responses against cariogenic bacteria. MDP is also known to induce the expression of MMP-1 in rheumatoid arthritis synovial fibroblasts[8)].

MDP has been shown to promote the production of inflammatory cytokines, such as IL-6 and IL-8, and exacerbate inflammation[7)]. The binding of MDP to NOD2 activates transforming growth factor beta-activated kinase 1 (TAK1) by recruiting receptor-interacting protein 2, and activated TAK1 then triggers the nuclear factor kappa-b (NF-κB) and MAP kinase (p38, ERK 1/2, and JNK) pathways, leading to the induction of innate immune responses[9-11)] .

Surface reaction-type prereacted glass-ionomer (S-PRG) fillers are a class of bioactive filler material

Received Sep 9, 2022: Accepted Nov 30, 2022
doi:10.4012/dmj.2022-201 JOI JST.JSTAGE/dmj/2022-201

produced by an acid-base reaction between porous silica glass-coated fluoroboroaluminosilicate glass fillers and a polyacrylic acid solution[9,10]. S-PRG filler has been shown to effectively suppress the activity of oral bacteria[12]. S-PRG filler eluate contains multiple ions, such as boron, fluoride, silicate, and strontium[13]. Strontium released from S-PRG filler was found to activate intracellular signaling *via* CaSR[14]. S-PRG fillers have been used in a number of dental materials. Recent studies demonstrated that pulp-capping material containing S-PRG fillers promoted the formation of reparative dentin in the exposed dental pulp of rats[15]. Based on these findings, S-PRG filler eluate appears to endow pulp capping with several beneficial properties, leading to reparative dentin. We previously demonstrated that S-PRG filler eluate enhanced the production of MMPs by human gingival fibroblast cells[16]. MMP-1 levels were increased by MDP, resulting in the development of inflammation, such as pulpitis[7]. However, limited information is currently available on the effects of S-PRG filler eluate in inflammatory pulp tissue. Therefore, we herein investigated whether a co-stimulation S-PRG filler eluate and MDP enhanced MAP kinase activity and promoted the production of MMP-1 by human dental pulp fibroblast-like cells (hDPFs). We also attempted to elucidate the underlying intracellular signaling pathway.

The purpose of this study was to investigate the effects of S-PRG filler eluate and MDP co-stimulation on MMP-1 production in hDPFs, and to investigate its intracellular signaling pathway.

MATERIALS AND METHODS

Cell culture

hDPFs were grown from explants from the healthy marginal gingiva of healthy donors. 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; Thermo Fisher Scientific, Waltham, MA, USA), 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) in an atmosphere of 5% CO_2-95% air at 37°C. The first subcultures were obtained 20 to 30 days later, maintained in an atmosphere of 5% CO_2-95% air at 37°C, 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. This study was approved by the Ethical Review Board of Osaka Dental University (Approval No.111112). Informed consent was obtained from all study participants, and the study was conducted according to the principles of the Declaration of Helsinki.

Preparation of S-PRG filler eluate

S-PRG fillers were prepared as previously reported[10]. S-PRG filler eluate was also prepared using a previously described method[10]. Briefly, α-MEM was mixed with S-PRG filler (average filler diameter of 1 μm) at a weight ratio of 1:1 (1 L:1,000 g), followed by gentle mixing with a tumbler mixer at room temperature for 24 h. After mixing, S-PRG filler was precipitated with a centrifuge and the supernatant was recovered. The supernatant collected was filtered through a chromatodisc (0.45 μm) to obtain S-PRG filler α-MEM eluate (S-PRG filler eluate). An elemental analysis of five ions (Na, Al, B, Sr, and Si) released from S-PRG filler was performed using inductively coupled plasma atomic emission spectroscopy (ICPS-8000, Shimadzu, Kyoto, Japan). Analyses were conducted after preparing calibration curves corresponding to each element (standard solution concentration; Na: 0, 0.5, 20, and 50 ppm; Al: 0, 0.5, 5, and 10 ppm; B: 0, 10, 50, and 100 ppm; Sr: 0, 5, 20, and 50 ppm; Si: 0, 0.5, 1, and 5 ppm). An elemental analysis was performed on F released from S-PRG filler using an ion electrode method with a fluoride electrode (9609BNWP, Thermo Fisher Scientific). Similarly, the concentration of F was assessed after preparing its calibration curves (standard solution concentrations: 0.1, 1, 5, and 10 ppm). Ion concentrations in S-PRG filler eluate were as follows: Al 14.2 ppm, B 1,894.9 ppm, Na 3,341.2 ppm, Si 11.0 ppm, Sr 924.0 ppm, and F 89.0 ppm (Table 1). All experiments used α-MEM medium eluting S-PRG filler components.

Table 1 Comparison of various ion concentrations between S-PRG filler eluate and α-MEM

	Elements/Ion concentration (ppm)								
	Al	B	Na	Si	Sr	F	Ca	P	K
α-MEM (Lot. KCF7035)	0	0	3,331.4	0	0	0	73.2	30.3	205.6
S-PRG filler eluate solution (Lot. 062023)	14.2	1,894.9	3,341.2	11.0	924.0	69.0	4.2	0	19.3

S-PRG filler eluate was prepared using the following method. α-MEM was mixed with S-PRG filler (1 μm average filler diameter) at a weight ratio of 1:1 (1 L:1,000 g), and the supernatant was collected. The collected supernatant was filtered to obtain S-PRG filler eluate. S-PRG, Surface pre-reacted glass ionomer; α-MEM, Alpha minimum essential medium

Cytotoxicity experiment

hDPFs were seeded on a 96-well plate at a density of 1×10^4 cells/well, and various concentrations of S-PRG filler eluates were incubated with or without 10 μg/mL MDP (InvivoGen, San Diego, CA, USA) at 37°C for 24 h in the presence of 5% CO_2. Cell proliferation was assessed by measuring absorbance (450 nm) on a 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 one-way analysis of variance (ANOVA). Each sample was tested in triplicate.

Western blotting

hDPFs were seeded onto 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 h. After the incubation, hDPFs were stimulated with S-PRG filler eluate (0, 001, 0.1, and 1%) or MDP (0, 1, 5, 10, and 20 μg/mL) for 48 h. Conditioned medium was collected, centrifuged to remove debris, concentrated up to 30-fold with Amicon Ultra (Merck, 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) 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 h. 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) was employed to visualize immunoreactive bands. Blot images were acquired using ChemiDoc MP (Bio-Rad, Hercules, CA, USA). In an experiment using NPS 2143 (Cayman Chemical, Ann Arbor, MI, USA), a selective and potent CaSR antagonist, hDPFs were cultured and treated without serum, as described in previous experiments. hDPFs were pretreated with 1 μM NPS 2143 for 1 h and then stimulated by the addition of 0.1% S-PRG filler eluate and/or 10 μg/mL MDP for 24 h. After the stimulation, MMP-1 and β-actin were similarly detected by Western blotting. In an experiment on ERK 1/2 phosphorylation, hDPFs were pretreated in the same manner. hDPFs were pretreated with 1 μM NPS 2143 for 1 h and were then stimulated by the addition of 0.1% S-PRG filler eluate and/or 10 μg/mL MDP for 10 min. 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 anti-phospho ERK 1/2 antibody (Cell Signaling Technology, Danvers, MA, USA) at 1000:1. Membranes were similarly treated with secondary antibodies and visualized phosphorylated bands were obtained using the ChemiDoc MP. The same membrane was stripped and reprobed with an anti-ERK 1/2 antibody (Cell Signaling Technology) at 1:1000. ChemiDoc MP software was used to quantify signal intensities. The bands of phosphorylated proteins and total proteins were quantitated using signal intensities, and their ratios were calculated.

RESULTS

Cytotoxicity of S-PRG filler eluate on hDPFs

We initially examined the effects of S-PRG filler eluate and MDP on cell proliferation. hDPFs were incubated in a 96-well plate (1×10^4 cells/well) with different concentrations of S-PRG filler eluate (0, 0.01, 0.1, and 1%) in the absence or presence of MDP (10 μg/mL) for 24 h. After the incubation, hDPFs were tested using the cell proliferation reagent WST-8. The proliferation and viability of hDPFs were examined using WST-8 (Fig. 1). None of the conditions showed a significant difference from the controls. In addition, there was no significant difference between the two groups other than the control. The results obtained revealed that MDP and S-PRG filler eluate were not cytotoxic and did not affect the proliferation or viability of hDPFs at the concentrations tested (Fig. 1).

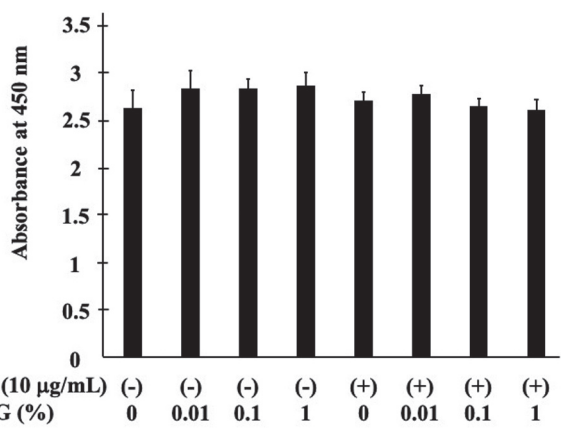

Fig. 1 Cytotoxicity of S-PRG filler eluate on hDPFs. Various dilutions of S-PRG filler eluate were incubated (0, 0.01, 0.1, and 1 %) with hDPFs in the presence or absence of MDP for 24 h. The effects of the co-culture of diluted S-PRG filler eluate and MDP on the proliferation of hDPFs was examined using the cell proliferation reagent WST-8. Data presented are representative of four independent experiments. No significant difference was observed from the control.

Effects of S-PRG filler eluate on the production of MMP-1 by hDPFs

We previously reported that S-PRG filler eluate-activated human gingival fibroblasts produced MMP-1[16]; therefore, we herein investigated whether S-PRG filler eluate affected the production of MMP-1 by hDPFs. When cells were cultured in the presence of 0.01, 0.1, and 1% S-PRG filler eluate, the dose-dependent production of MMP-1 was observed. The production of MMP-1 in hDPFs was enhanced by the stimulation with 1% S-PRG filler eluate, but not by 0.1% or less (Fig. 2). Neither the total amount of β-actin nor the activation status was altered by the stimulation with S-PRG filler eluate. These results indicated that 0.1% S-PRG filler eluate did not promote the production of MMP-1 by hDPFs.

Co-stimulation effects of a low concentration of S-PRG filler eluate on the production of MMP-1 in MDP-treated hDPFs

When hDPFs were cultured in the presence of 1, 5, 10, and 20 μg/mL MDP, the dose-dependent production of MMP-1 was observed, with a peak at 10 μg/mL (Fig. 3A). The results obtained showed that 0.1% S-PRG filler eluate did not promote the production of MMP-1 by hDPFs (Fig. 2). Therefore, we investigated the co-stimulatory effects of 0.1% S-PRG filler eluate in hDPFs treated with 10 μg/mL MDP. The co-stimulation with 0.1% S-PRG filler eluate and MDP resulted in the production of higher levels of MMP-1 than the MDP treatment alone (Fig. 3B). One of the major components of S-PRG filler eluate is strontium ions (Table 1), which activate intercellular signaling *via* the membrane-bound ion channel, CaSR[17]. NPS 2143, an orally active calcilytic agent, is a selective and potent CaSR antagonist. NPS 2143 attenuated the increases induced in the production of MMP-1 by the co-stimulation with 0.1% S-PRG filler eluate and MDP in hDPFs (Fig. 3B). These results indicate that the increases induced in MMP-1 production by the co-stimulation with S-PRG filler eluate were due to strontium.

S-PRG filler eluate phosphorylated ERK 1/2 in hDPFs

Previous findings showed that NOD2 activated ERK 1/2[18] and that the up-regulation of MMP-1 depended on the ERK 1/2 signaling pathway[19]. The present results demonstrated that the production of MMP-1 involved CaSR (Fig. 3B). Therefore, we investigated whether the activation of ERK 1/2 involved CaSR in hDPFs stimulated with S-PRG filler eluate. ERK 1/2 phosphorylation in hDPFs was enhanced by 0.1% S-PRG filler eluate (Fig. 4). NPS 2143 attenuated the increases induced in the

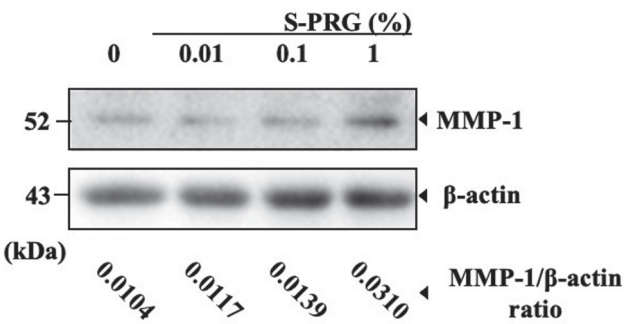

Fig. 2 Effects of various concentrations of S-PRG filler eluate on MMP-1 production by hDPFs.
hDPFs were stimulated by various concentrations of S-PRG filler eluate for 24 h, and the supernatant was concentrated. Western blotting was used to evaluate MMP-1 production and β-actin (as a control) in hDPFs. The bands of phosphorylated proteins and total protein ratios were calculated. Data presented are representative of four independent experiments. hDPFs, human dental pulp fibroblast-like cells; MMP, matrix metalloproteinase; S-PRG, Surface pre-reacted glass ionomer

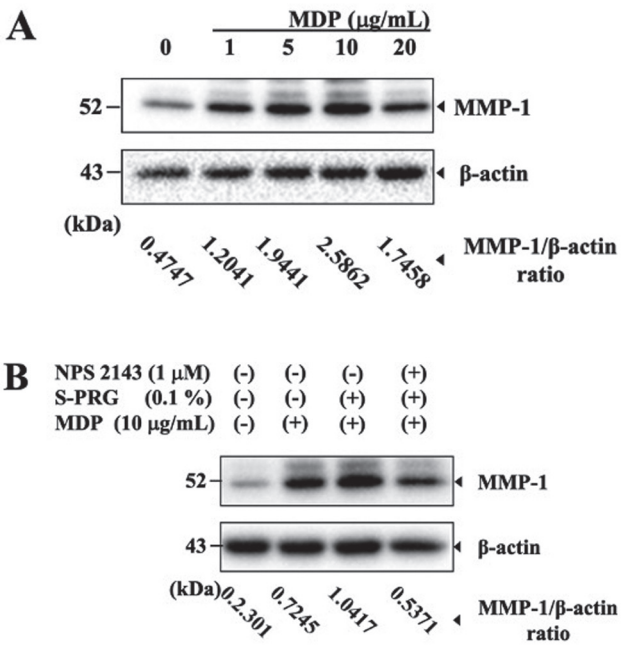

Fig. 3 Role of S-PRG filler elution in MMP-1 production by hDPFs co-stimulated with MDP.
A: hDPFs were stimulated with various concentrations of MDP for 24 h, and the supernatant was concentrated. Western blotting was used to evaluate MMP-1 production and β-actin (as a control) in hDPFs. B: hDPFs were pretreated with 1 μM NPS 2143 for 1 h and then co-stimulated with 0.1% S-PRG filler eluate and 10 μg/mL MDP for 24 h. Western blotting was used to evaluate MMP-1 production and β-actin (as a control) in hDPFs. The bands of phosphorylated proteins and total protein ratios were calculated. Data presented are representative of four independent experiments. hDPFs, human dental pulp fibroblast-like cells; MDP, muramyl dipeptide; MMP, matrix metalloproteinase; S-PRG, Surface pre-reacted glass ionomer

phosphorylation of ERK 1/2 by 0.1% S-PRG filler eluate (Fig. 4). The total amount of ERK 1/2 proteins was not affected under any of the experimental conditions used (Fig. 4). These results indicate that 0.1% S-PRG filler eluate enhanced the phosphorylation of ERK 1/2, but not the production of MMP-1.

The ERK 1/2 signaling pathway was a downstream target of CaSR in MDP-treated hDPFs

We then examined the role of strontium in the increases induced in MMP-1 production by the co-stimulation with S-PRG filler eluate. MDP induced the phosphorylation of ERK 1/2 in hDPFs (Fig. 5). The co-stimulation with 0.1% S-PRG filler eluate and MDP enhanced ERK 1/2 phosphorylation more than MDP alone (Fig. 5). When

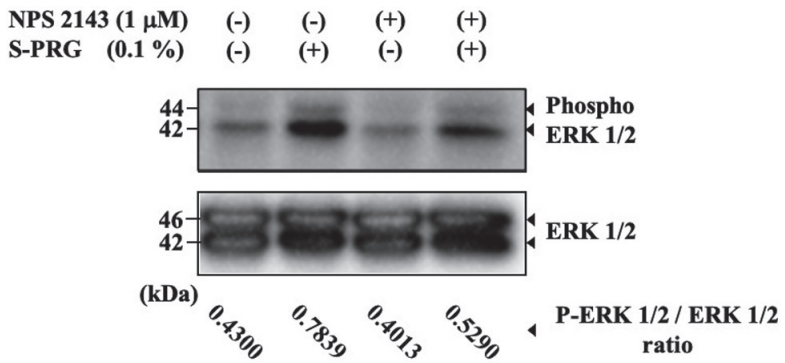

Fig. 4 Effects of NPS 2143 on ERK 1/2 phosphorylation in S-PRG filler eluate-stimulated hDPFs.
hDPFs were pretreated with 1 μM NPS 2143 for 1 h and then stimulated with 0.1% S-PRG filler eluate for 10 min. Phospho-ERK 1/2 and ERK 1/2 expression levels were evaluated by Western blotting. The bands of phosphorylated proteins and total protein ratios were calculated. Data presented are representative of four independent experiments. hDPFs, human dental pulp fibroblast-like cells; MDP, muramyl dipeptide; S-PRG, Surface pre-reacted glass ionomer, ERK 1/2; extracellular signal-regulated kinase 1/2

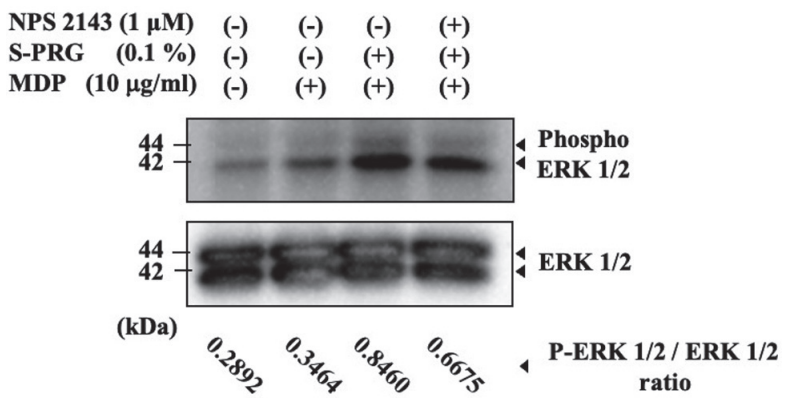

Fig. 5 Effects of NPS 2143 on ERK 1/2 phosphorylation in hDPFs co-stimulated with MDP and S-PRG.
hDPFs were pretreated with 1 μM NPS 2143 for 1 h and then co-stimulated with 0.1% S-PRG filler eluate and 10 μg/mL MDP for 10 min. Phospho-ERK 1/2 and ERK 1/2 expression levels were evaluated by Western blotting. The bands of phosphorylated proteins and total protein ratios were calculated. Data presented are representative of four independent experiments. hDPFs, human dental pulp fibroblast-like cells; MDP, muramyl dipeptide; S-PRG, Surface pre-reacted glass ionomer, ERK 1/2; extracellular signal-regulated kinase 1/2

hDPFs were cultured with the CaSR antagonist, NPS 2143, the phosphorylation of ERK 1/2 was suppressed by NPS 2143 in hDPFs co-stimulated with S-PRG filler eluate and MDP (Fig. 5). The total amount of ERK 1/2 proteins was not affected under any of the experimental conditions used (Fig. 5). These results showed that the activity of ERK 1/2 was a downstream target of CaSR in S-PRG filler eluate signaling pathways.

DISCUSSION

The NOD protein, NOD2, which is a member of the intracellular NOD-like receptor family, detects conserved motifs in bacterial peptidoglycans and promotes their clearance through the activation of a pro-inflammatory transcriptional program and other innate immune pathways[20]. NOD2 is constitutively expressed in hDPFs and plays a role in pulpal immune responses, whereas NOD1 does not[7,21]. NOD1/2 have been shown to drive the activation of the MAP kinase and NF-κB pathways, leading to pro-inflammatory cytokine production in human dental pulp[7,22].

S-PRG fillers are a class of bioactive filler materials produced by an acid-base reaction between porous silica glass-coated fluoroboroaluminosilicate glass fillers and a polyacrylic acid solution, by which a glass-ionomer phase is formed between the surface phase and glass core phases[13]. S-PRG fillers have been incorporated into a number of dental materials, including pulp capping[15]. In the present study, we demonstrated that signaling pathways activated by a low concentration of S-PRG filler eluate enhanced the production of MMP-1 by MDP-treated hDPFs, which is important for investigating the effects of S-PRG filler eluate on pulpitis, such as bacterial infection. We previously reported that 10% S-PRG filler eluate affected the proliferation of human gingival fibroblasts, whereas 1% or less did not[23]. We initially examined the effects of S-PRG filler eluate in combination with 10 μg/mL MDP on the proliferation of hDPFs. None of the concentrations tested (0.01, 0.1, and 1%) were toxic and had no effect on the proliferation of hDPFs treated with 10 μg/mL MDP (Fig. 1). hDPFs were examined using a dilution ratio of 100-fold (1%) or more of S-PRG filler eluate with MDP.

MMPs are zinc-dependent endopeptidases that play an integral role in the maintenance of normal organ and tissue homeostasis. MMP-1 is regarded as a key enzyme in the degradation of the triple helix of collagen type I[24]. Previous studies reported that several MMPs were expressed in pulpitic tissue and played a role in pulp tissue destruction[25]. We investigated whether S-PRG filler eluate increased the production of MMP-1. The production of MMP-1 by hDPFs was promoted by 1% S-PRG filler eluate, but not by 0.1% or less (Fig. 2). When cells were cultured in the presence of MDP, the dose-dependent production of MMP-1 was noted, with a peak at 10 μg/mL MDP (Fig. 3A). Although MMP-1 production was not induced by 0.1% S-PRG filler eluate, further studies are warranted to examine the effects of low concentrations of S-PRG filler eluate under inflammatory conditions, such as those induced by NOD1/2. We then examined the effects of the co-stimulation with 0.1% S-PRG filler eluate and 10 μg/mL MDP on MMP-1 production by hDPFs. Although 0.1% S-PRG filler eluate did not promote the production of MMP-1, MMP-1 levels were higher following the co-stimulation with 0.1% S-PRG filler eluate and MDP than with MDP alone (Fig. 3B). The present results confirmed that the co-stimulation with MDP enhanced MMP-1 production, whereas a low concentration of S-PRG filler eluate alone had no effect. Strontium ions have been shown to promote the differentiation and mineralization of osteoblasts[17]. In human monocytes, strontium reduced LPS-induced enhancements in the production of TNF-α, IL-6, and IL-8[11]. Therefore, strontium influences various cellular bioactivities. Since there are six types of ions, including strontium, in S-PRG filler eluate[13], we investigated whether strontium in S-PRG filler eluate affected the production of MMP-1 in hDPFs. CaSR are G protein-coupled receptors that are activated by an increase in extracellular calcium or strontium[14]. The activation of CaSR is known to induce the odontoblast differentiation of human dental pulp cells and the mineralization of mouse osteoblastic MC3T3-E1 cells[26]. NPS 2143 is a selective and potent CaSR antagonist. The combination of NPS 2143 with a 0.1% S-PRG filler effluent stimulation did not affect MMP-1 production, similar to the 0.1% S-PRG filler effluent stimulation alone (data not shown). In the present study, NPS 2143 suppressed the production of MMP-1 induced by the co-stimulation with S-PRG filler eluate and MDP in hDPFs (Fig. 3B). These results suggest that NPS 2143 inhibited the CaSR stimulation mediated by strontium in S-PRG filler eluate, but not by calcium in the medium. Therefore, strontium likely functions as a co-stimulatory factor of MMP-1 production by hDPFs. A previous study reported that NPS 2143 down-regulated ALP and IBSP mRNA expression in osteoblasts cultured in an eluate from root canal sealers containing S-PRG filler[17]. Strontium bound to CaSR and up-regulated several intracellular signaling pathways, including MAP kinases, such as p38 and ERK 1/2[17,26,27]. Moreover, the activation of MAP kinase *via* NOD-2 was demonstrated in murine macrophages, in which p38, ERK 1/2, and JNK were phosphorylated after a stimulation with MDP[17]. We previously demonstrated that ERK 1/2 was involved in MMP-1 production by human gingival fibroblasts stimulated with S-PRG filler eluates[16]. Therefore, in the present study, we investigated changes in ERK1/2 phosphorylation upon a co-stimulation with MDP and S-PRG filler eluate. The CaSR-mediated phosphorylation of ERK 1/2 in the presence of 0.1% S-PRG filler eluate was assessed. Since the phosphorylation of ERK 1/2 was similar to that of the control only in the presence of NPS 2143 based on the results shown in Fig. 4, calcium in the medium did not appear to affect ERK 1/2 phosphorylation *via* CasR. On the other hand, the phosphorylation of ERK 1/2 enhanced by 0.1% S-PRG filler eluate was suppressed by NPS 2143. This suggests that strontium in S-PRG filler eluate was involved in ERK 1/2 phosphorylation *via* CasR.

However, the suppression of ERK 1/2 phosphorylation by NPS2143 was not to the same extent as that in the control. Since S-PRG filler eluate contains five ions other than strontium, these ions may play a role in the phosphorylation of ERK 1/2. In addition, to confirm the involvement of ERK 1/2 on MMP-1 production, in future studies we will investigate the effects of ERK-specific inhibitors on MMP-1 production. The 0.1% S-PRG filler eluate induced the phosphorylation of ERK1/2, but MMP-1 production by 0.1% S-PRG filler eluate could not be confirmed by Western blotting. Western blotting is primarily a binary comparison method for demonstrating a change in a protein of interest. Accordingly, western blotting may not have captured the slight increase in MMP-1 production by the 0.1% S-PRG filler eluate. In future studies, we will consider quantitative analysis of MMP-1 using ELISA. We examined the phosphorylation of ERK 1/2 *via* CaSR upon a co-stimulation with 0.1% S-PRG filler eluate and 10 μg/mL MDP. Based on the results shown in Fig. 5, strontium in S-PRG filler eluate is likely involved in enhancing ERK 1/2 phosphorylation by the co-stimulation with S-PRG filler eluate and MDP. Furthermore, the results in Fig. 4 indicated that calcium in the medium did not affect ERK 1/2 phosphorylation *via* CasR in the experiment shown in Fig. 5. Moreover, similar to the results in Fig. 4, the suppression of ERK 1/2 phosphorylation by NPS 2143 was not completely suppressed to the level of MDP alone, suggesting the involvement of ions other than strontium. Based on the results in Table 1, the estimated strontium concentration in 0.1% S-PRG filler eluate is 0.924 ppm (10.55 μM). Buache *et al*. reported that a stimulation with 10 nM strontium alone did not affect inflammatory cytokine production by human monocytes, whereas a co-stimulation with LPS and 10 nM strontium reduced LPS-stimulated increases in TNF-α, IL-6, and IL-8 production[11]. This study[11] also demonstrated that even strontium at a concentration of 0.94 ppm (10.73 μM) in 0.1% S-PRG filler eluate was sufficient to affect biological activity, which supports our hypothesis that the enhanced production of MP1 by the co-stimulation with 0.1% S-PRG filler eluate and MDP is an effect of strontium.

Collectively, the present results showed that a low concentration of S-PRG filler eluate (0.1%), which alone exerted no effects, enhanced MDP-stimulated MMP-1 production when co-stimulated with MDP. In addition, this co-stimulatory enhancement in the production of MMP-1 was attributed to the activity of ERK 1/2, downstream targets of CaSR. These results also indicate that S-PRG filler is a bioactive pulp capping agent with the ability to promote pulp tissue remodeling. Since inflammation may be exacerbated depending on the concentration of S-PRG filler eluate, it is important to investigate the signaling pathways and production of other MMPs in hDPFs.

CONCLUSION

The present study demonstrated that a co-stimulation with S-PRG filler eluate containing strontium ions and MDP activated ERK 1/2 signaling pathways *via* CaSR and promoted MMP-1 production. These results indicate that even without S-PRG filler, cellular bioactivity may be induced in the presence of S-PRG filler eluate containing multiple ions eluted from S-PRG filler. The present study also provides useful information for the development of novel bioactive S-PRG filler-containing pulp capping agents with the ability to promote pulp tissue remodeling.

ACKNOWLEDGMENTS

We are grateful to Dr. Mao from the Graduate School 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 (a Grant-in-Aid for Scientific Research (C)) and Osaka Dental University Research Funds (22-02).

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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