# Down-regulation of Inflammatory Mediator Synthesis and Infiltration of Inflammatory Cells by MMP-3 in Experimentally Induced Rat Pulpitis

*Koyo Takimoto, DDS,* Nobuyuki Kawashima, DDS, PhD,* Noriyuki Suzuki, DDS, PhD,* Yu Koizumi, DDS, PhD,* Mioko Yamamoto, DDS,* Misako Nakashima, DDS, PhD,† and Hideaki Suda, DDS, PhD**

## Abstract

**Introduction:** Matrix metalloproteinase (MMP)-3 is a member of the MMP family that degrades the extracellular matrix. Application of MMP-3 to injured pulp tissue induces angiogenesis and wound healing, but its anti-inflammatory effects are still unclear. Here, we evaluated the anti-inflammatory functions of MMP-3 *in vitro* and *in vivo*. **Methods:** Nitric oxide and inflammatory mediator synthesis in macrophages activated by lipopolysaccharide (LPS) was measured in the presence or absence of MMP-3. The mouse Mmp3 (mMmp3) expression vector containing full length cDNA sequence of mMmp3 or cDNA sequence of mMmp3 missing the signal peptide and pro-peptide regions was transfected to RAW264, a mouse macrophage cell line, and NO synthesis and inflammatory mediator expression were evaluated. Pulpal inflammation was histologically and immunohistochemically evaluated in a rat model of incisor pulpitis induced by the application of LPS for 9 hours in the presence or absence of MMP-3. **Results:** NO and pro-inflammatory mediator synthesis promoted by LPS was significantly down-regulated by MMP-3 *in vitro*. The full length of mMmp3 down-regulated the LPS-induced NO synthesis and chemical mediator mRNA expression, however the mMmp3 missing the signal peptide failed to block the NO synthesis induced by LPS. The numbers of major histocompatibility complex class II+ and CD68+ cells, which infiltrated into the rat incisor pulp tissues in response to the topical application of LPS, were significantly decreased by the application of MMP-3 *in vivo*. **Conclusions:** These results indicate that MMP-3 possesses anti-inflammatory functions, suggesting its potential utility as an anti-inflammatory agent for pulpal inflammation. *(J Endod 2014;40:1404–1409)*

## Key Words

Cytokine(s), inflammation, matrix metalloproteinase-3, pulp biology, pulpitis

Pulpitis is an inflammatory disease after the development of caries or dental traumatic lesions, and it is caused by the immunological host defense reaction activated by bacterial infection (1). Bacterial invasion in the pulp tissue activates resident immunocompetent cells and induces infiltration of immunocompetent cells. These cells produce large amounts of chemical mediators that typically characterize pulpal pathosis. The initial and fundamental response against exogenous stimuli in the pulp tissue is innate immunity, and macrophages are typical members of this innate immunity (2). After the development of carious lesions, the amount of exogenous stimuli overcomes the removal capacity of resident macrophages, leading to infiltration of activated macrophages and/or neutrophils, which characterizes the pathogenesis of acute pulpitis. Various proinflammatory mediators, such as nitric oxide (NO), interleukin (IL)-1, IL-6, and tumor necrosis factor alpha (TNF-$\alpha$), are produced by macrophages in the pulpitis (1, 3). Prostaglandins synthesized by cyclooxygenase 2 (Cox 2) are also produced in the inflamed pulp (4). These mediators characterize pulpal pathosis and determine the establishment of pulpitis. Overproduction of these proinflammatory mediators further stimulates their production, which induces a vicious cycle. Pulp tissue is encased in dentin hard tissue, resulting in low compliance (5), and it is essential to regulate these overexpressed chemical mediators to avoid pulpal necrosis. In addition, the control of inflammation is very important for pulp regeneration (6) and regenerative endodontics (7).

Matrix metalloproteinase (MMP)-3 is a member of the MMP family, typical proteinases that degrade the extracellular matrix. MMPs affect migration, differentiation, growth, inflammatory processes, angiogenesis, and apoptosis (8). Furthermore, MMPs are involved in various physiological and pathological processes such as joint disorders, cancer, and coronary heart disease (9). However, MMP-3 is also reported to inactivate proinflammatory mediators (10), enhance clearance of inflammatory cells, and regulate inflammatory conditions (11, 12). These reports indicate that MMPs are involved in both tissue destruction and wound healing.

MMP-3 expression has been observed in healthy pulp tissue (13), and it is involved in remodeling of the dentin matrix (14), suggesting that MMP-3 may be related to the maintenance of pulpal homeostasis. Furthermore, MMP-3 expression has been observed in inflamed (13) and injured (15) pulp tissue. Recently, dental pulp stem

From the *Department of Pulp Biology and Endodontics, Division of Oral Health Sciences, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan; and †Department of Dental Regenerative Medicine, Center of Advanced Medicine for Dental and Oral Diseases, National Center for Geriatrics and Gerontology, Research Institute, Aichi, Japan.

Address requests for reprints to Dr Nobuyuki Kawashima, Department of Pulp Biology and Endodontics, Division of Oral Health Sciences, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Bunkyo-ku, Tokyo, 113-8549 Japan. E-mail address: kawashima.n.endo@tmd.ac.jp

0099-2399/$ - see front matter



http://dx.doi.org/10.1016/j.joen.2014.04.001

cells were reported to synthesize large amounts of MMP-3 (16). Moreover, the application of MMP-3 to injured pulp tissue induces angiogenesis (17). These reports suggest that MMP-3 may be involved in wound healing of pulp tissue. However, the anti-inflammatory effects have not been fully evaluated for MMP-3. In this study, we evaluated the anti-inflammatory properties of MMP-3 in pulpal inflammation *in vitro* and *in vivo*.

## Materials and Methods

### Nitric Oxide Assay and Bead Array

All animal experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (#0120156A). Six-week-old male Wistar rats ($N = 2$; Clea Japan, Tokyo, Japan) was used as the source of macrophages. Resident peritoneal macrophages were obtained after intraperitoneal injection of a 20 mL cold saline solution. Macrophages were cultured in RPMI-1640 (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% FBS (60°C heat-inactivated; Thermo Scientific HyClone, Logan, UT, USA) and a 1% penicillin-streptomycin-amphotericin B solution (Wako).

Macrophages ($3 \times 10^4$ cells/well in 96-well plate; NO synthesis, $1.6 \times 10^5$ cells/well in 96-well plates; bead array) were stimulated with LPS (100 ng/mL; E.Coli 0111:B4; Sigma-Aldrich, St Louis, MO) in the presence or absence of MMP-3 (100 ng/mL; EMD Millipore; Billerica, MA) for 20 hours. Non-LPS-stimulated macrophages were used as a control. NO synthesis was estimated by the accumulation of nitrite that was measured by the Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethylene-diamine dihydrochloride, and 2.5% phosphoric acid; Sigma-Aldrich). The Bradford protein assay was used to measure the total protein (Protein Quantification Kit-Rapid, Dojindo, Kumamoto, Japan). Cytokines in the culture medium were measured by Bio-Plex Suspension Array System (Bio-Rad Laboratories, Hercules, CA) using Rat Cytokine/Chemokine magnetic beads panel 96-well plate assay kit (EMD Millipore).

### Construction of Eukaryotic Expression Vectors and Transfection

The full-length (FL) open reading frame of mouse (m)Mmp3 complementary DNA (cDNA) was subcloned into the pEF-Dest51, an eukaryotic expression vector for synthesis of C-terminally V5- and 6xHis-tagged proteins (Life Technologies, Carlsbad, CA) to create pEF-mMmp3FL. mMmp3 cDNAs with the original signal sequence and propeptide sequence removed was also subcloned into pEF-Dest51 to create pEF-mMmp3SP&PP-. An enhanced green fluorescent protein (EGFP) expression vector was used as a control (pEF-EGFP). Protein expression was detected by Western blotting using an anti-V5 antibody (1:2000, clone: V5005; Nacalai Tesque, Kyoto, Japan), which was probed with an HRP-conjugated anti-mouse IgG secondary antibody (1:1000, Vector Laboratories, Burlingame, CA), and chemiluminescence HRP substrate (Immobilon, EMD Millipore). Chemiluminescent images were detected by LAS3000 (Fuji Film, Tokyo, Japan). RAW264, a mouse macrophage-like clonal cell line (Riken BRC, Tsukuba, Japan) cells were cultured in High Glucose D-MEM (Wako) supplemented with 10% FBS (60°C heat-inactivated; Thermo Scientific HyClone) and a 1% penicillin-streptomycin-amphotericin B solution (Wako) at 37°C with 5% $CO_2$. The expression vectors were transfected into RAW264 cells using FuGENE (Roche, Basel, Switzerland). After 48 hours, LPS (100 ng/mL; *E. coli* 0111:B4, Sigma-Aldrich) was added to the transfected cells ($2 \times 10^5$ cells/well in 96-well plate; NO synthesis, $1 \times 10^6$ cells/well in 24-well plate; RT-PCR) which were cultured for 20 hours. NO synthesis was measured by the Griess reagent as described previously.

### RT-PCR

Total RNA was extracted from RAW264 cells with Quick Gene Mini-80 (Fuji film). cDNA was synthesized from total RNA (300 ng) using RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific Inc., Waltham, MA) and an oligo (dT) 18. Quantitative RT-PCR was performed using GoTaq qPCR Master Mix (Promega, Madison, WI) on a CFX96 (Bio-Rad). Quantitative RT-PCR amplification was performed at 95°C for 15 seconds, and 60°C for 60 seconds for 45 cycles. Specific primers and product sizes (in parentheses) were as follows: $\beta$-actin: 5′-AGGGAAATCGTGCGTGACAT-3′ and 5′-AACCGCTCGTTGCCAATAGT-3′ (130 bp); IL-1$\beta$: 5′-ACCCAAGCACCTTCTTTTCC-3′ and 5′-GTTTGGGATCCACACTCTCC-3′ (351 bp); IL-6: 5′-ATGTTGTTGACAGCCACTGC-3′ and 5′-AAACGGAACTCCAGAAGACC-3′ (314 bp); Cox2: 5′-AGTATCAGAACCGCATTGCC-3′ and 5′-TAAGGTTTCAGGGAGAAGCG-3′ (310 bp). Primers for IL-1$\beta$, 6 and Cox2 were designed for rat targets, and slight difference of nucleic acid sequences occurred. However, there was no difference at 3′ end of their sequence, and they work well on mice samples.

### Induction of Pulpitis

Six-week-old male Wistar rats ($N = 18$; Clea Japan) were given free access to food and water. After anesthesia induced by an intraperitoneal injection of a mixture of ketamine hydrochloride (2.5 mg/100 g body weight; Sankyo, Tokyo, Japan) and sodium pentobarbital (2.5 mg/100 g body weight [Somnopentyl; Kyoritsu Seiyaku Corp, Tokyo, Japan]), 2 mm of mandibular left and right incisor crowns were removed by a diamond disc to expose the pulp chamber. After disinfection with 70% ethanol, the coronal pulp chamber was enlarged by sterilized K-files up to #40. LPS (10 $\mu$g/mL, 1 $\mu$L), sterile saline (1 $\mu$L), or LPS (10 $\mu$g/mL, 1 $\mu$L) plus MMP-3 (100 $\mu$g/mL, 1 $\mu$L) were applied to the exposed upper incisor pulps with a sterile paper point (Johnson & Johnson, New Brunswick, NJ). The cavity was sealed with Caviton (GC, Tokyo, Japan). Rats were sacrificed under anesthesia at 9 hours postoperatively, and the upper incisors were extracted. Infiltration of macrophages in response to LPS has been reported to peak at 9 hours in a previous study (18).

### Immunohistochemistry

Pulp tissues were fixed with 4% paraformaldehyde at 4°C overnight and then decalcified with 15% EDTA at 4°C for 4 weeks. Samples were embedded in Tissue-Tek O.C.T. Compound (Sakura, Tokyo, Japan) and quickly frozen in hexane (Wako) cooled by dry ice. Immunohistochemical staining was performed on sections (7-$\mu$m thickness) with mouse antirat major histocompatibility complex (MHC) class II RT1B (1:10,000, clone: OX6; Serotec, Oxford, UK) and mouse antirat CD68 (1:100, clone: ED1; Serotec) antibodies by incubation at 4°C overnight. Sections were then incubated with biotinylated horse anti-mouse IgG (1:1000; Vector Laboratories) at room temperature for 30 minutes followed by avidin-biotin-peroxidase complex (VECTASTAIN Elite ABC Reagent, R.T.U, Vector Laboratories) at room temperature for 30 minutes. Colorization was performed with 3,3′-diaminobenzidine (DAB; ImmPACT DAB Peroxidase Substrate, Vector Laboratories). Sections were counterstained with methyl green (Muto Pure Chemicals, Tokyo, Japan).

Positively stained inflammatory cells on 3 typical sections from each sample were counted in 10 high-power fields (×20: objective, ×10: ocular) of the dental pulp tissue excluding abscesses, blood vessels, and dentin. The 3 typical sections were selected at intervals of 10 serial sections containing the majority of the dental pulp tissue. The average number of cells in the 10 visual fields of selected sections was regarded as representative.