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Journal of Cellular Biochemistry 113:1348–1358 (2012)

Journal of Cellular Biochemistry

# MMP-3 Provokes CTGF/CCN2 Production Independently of Protease Activity and Dependently on Dynamin-Related Endocytosis, Which Contributes to Human Dental Pulp Cell Migration

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## ABSTRACT

Matrix metalloproteinase-3 (MMP-3) expression is promoted after pulpotomy, and application of MMP-3 to dental pulp after pulpotomy accelerates angiogenesis and hard tissue formation. However, the mechanism by which MMP-3 promotes dental pulp wound healing is still unclear. Connective tissue growth factor/CCN family 2 (CTGF/CCN2), a protein belonging to the CCN family, is considered to participate in wound healing, angiogenesis, and cell migration. In this study, we examined the involvement of CTGF/CCN2 in MMP-3-induced cell migration in human dental pulp (fibroblast-like) cells. In human dental pulp cells, MMP-3 promoted cell migration, but this effect was clearly blocked in the presence of anti-CTGF/CCN2 antibody. MMP-3 provoked mRNA and protein expression and secretion of CTGF/CCN2 in a concentration- and time-dependent manner. The MMP-3 inhibitor NNGH failed to suppress MMP-3-induced CTGF/CCN2 protein expression. The potent dynamin inhibitor dynasore clearly inhibited MMP-3-induced CTGF/CCN2 expression. These results strongly suggest that MMP-3 induces CTGF/CCN2 production independently of the protease activity of MMP-3 and dependently on dynamin-related endocytosis, which is involved in cell migration in human dental pulp cells. J. Cell. Biochem. 113: 1348–1358, 2012. 

**KEY WORDS:** MMP-3; CTGF/CCN2; DYNAMIN; ENDOCYTOSIS; CELL MIGRATION; DENTAL PULP

Direct pulp capping has been used to treat dental pulp that has become exposed due to deep dental caries or traumatic injury. Generally, calcium hydroxide materials or mineral trioxide aggregate (MTA) are adopted for this purpose because of their ability to induce dentin bridge formation, which protects the dental pulp from various stimuli. However, these pulp capping materials can trigger mild inflammation and a necrotic layer in the subjacent pulp tissue [Schröder, 1985; Kuratate et al., 2008]. This process of reparative dentinogenesis is considered to be a nonspecific response.

Matrix metalloproteinases (MMPs) are a family of endopeptidases that play an important role in the process of tissue remodeling by extracellular matrix (ECM) degradation [Chakraborti et al., 2003; Visse and Nagase, 2003; Nagase et al., 2006]. On the basis of their substrate specificity, they are classified into collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs

Abbreviations used: MMP, matrix metalloproteinase; CTGF/CCN2, connective tissue growth factor/CCN family 2; MTA, mineral trioxide aggregate; ECM, extracellular matrix; bFGF, basic fibroblast growth factor; HUVECs, human umbilical vein endothelial cells; FBS, fetal bovine serum; α-MEM, α-minimal essential medium; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF-β, transforming growth factor-β; $[Ca^{2+}]i$, intracellular $Ca^{2+}$ concentration.

Grant sponsor: Japan Society for the Promotion of Science (JSPS); Grant numbers: 20592239, 22791846; Grant sponsor: The Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT); Grant number: 2011-2013 (S1001024).

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Received 14 July 2011; Accepted 18 November 2011 • DOI 10.1002/jcb.24007 • © 2011 Wiley Periodicals, Inc.
Published online 1 December 2011 in Wiley Online Library (wileyonlinelibrary.com).

(MT-MMPs) [Visse and Nagase, 2003; Nagase et al., 2006]. These MMPs are generated by a wide variety of cells such as fibroblasts, endothelial cells, osteoblasts, macrophages, lymphocytes, and neutrophils. Matrix metalloproteinase-3 (MMP-3), also termed stromelysin-1, has been reported to play a role in the organization of dermal wound healing [Bullard et al., 1999] and myocardial wound healing [Mukherjee et al., 2005], as well as the degradation of collagens (types III, IV, V, and IX), gelatin, aggrecan, versican, perlecan, decorin, proteoglycan, fibronectin, laminin, and osteonectin [Chakraborti et al., 2003]. In fact, MMP-3 degrades perlecan in human umbilical arterial endothelial-cell basement membranes to release basic fibroblast growth factor (bFGF) [Whitelock et al., 1996]. It is also capable of activating the other MMPs, including MMP-1, -7, -8, -9, -13 [Chakraborti et al., 2003]. MMP-3 is synthesized as a proform and activated by proteases such as plasmin, kallikrein, chymase, and tryptase [Chakraborti et al., 2003]. In the rat dental pulp, it is known that MMP-3 expression is promoted after pulpotomy. In addition, the application of MMP-3 to pulp after pulpotomy accelerates angiogenesis and hard tissue formation [Zheng et al., 2009]. Therefore, it is expected that MMP-3 would enhance the healing of injured dental pulp, suggesting that it could be applied as a new pulp capping material. However, the mechanism by which MMP-3 promotes pulp wound healing is still unclear.

Connective tissue growth factor/CCN family 2 (CTGF/CCN2) is a cysteine-rich secretory protein belonging to the CCN family, which also includes cysteine-rich 61 (CYR61/CCN1), nephroblastoma overexpressed (NOV/CCN3), Wnt-induced secreted protein-1 (WISP-1/CCN4), WISP-2 (CCN5), and WISP-3 (CCN6) [Lau and Lam, 1999; Moussad and Brigstock, 2000; Holbourn et al., 2008]. A prototypical CCN protein comprises four functional domains: insulin-like growth factor binding protein-like module (IGFBP), von Willebrand factor type C repeat module (VWC), thrombospodin type-1 repeat module (TSP-1), and C-terminal cystine knot-containing module (CT), but only CCN5 lacks a CT module [Lau and Lam, 1999; Moussad and Brigstock, 2000; Holbourn et al., 2008]. CTGF/CCN2 has been considered to participate in many biological events, including fracture healing [Nakata et al., 2002], cell migration [Bradham et al., 1991; Babic et al., 1999; Shimo et al., 1999; Guo et al., 2009], angiogenesis [Shimo et al., 1999; Nakata et al., 2002], endochondral and intramembranous ossification [Nakata et al., 2002; Kadota et al., 2004], cell proliferation [Shimo et al., 1999; Asano et al., 2005], ECM production [Frazier et al., 1996], and cell adhesion [Kireeva et al., 1997; Babic et al., 1999]. Human CTGF/CCN2 was originally identified as a novel growth factor in culture medium of human umbilical vein endothelial cells (HUVECs) [Bradham et al., 1991]. Recent studies have shown that this multifunctional protein is expressed in mouse embryo [Surveyor and Brigstock, 1999], especially developing tooth germs [Shimo et al., 2002] and oral epithelia [Kireeva et al., 1997], and is synthesized in various cell types such as fibroblasts [Ryseck et al., 1991; Frazier et al., 1996; Kadota et al., 2004], chondrocytes [Nakata et al., 2002; Kadota et al., 2004; Eguchi et al., 2008], osteoblasts [Nakata et al., 2002; Kadota et al., 2004], platelets [Kubota et al., 2004], and retinal pigment epithelium cells [Guo et al., 2009]. In addition, CTGF/CCN2 is synthesized in gingival fibroblasts and periodontal ligament cells [Asano et al., 2005; Takeuchi et al., 2009], and has been reported to induce proliferation and differentiation in murine periodontal ligament-derived cell line [Asano et al., 2005].

In the present study, we demonstrate that MMP-3 stimulates CTGF/CCN2 expression and secretion independently of the protease activity and dependently on dynamin-related endocytosis, which is involved in cell migration in human dental pulp cells.

## MATERIALS AND METHODS

### MATERIALS

Fetal bovine serum (FBS), α-minimal essential medium (α-MEM), fungizone and trypsin were purchased from GIBCO BRL Life Technologies (Tokyo, Japan). Penicillin G and kanamycin were purchased from Meiji Seika (Tokyo, Japan). Human MMP-3 (recombinant) was obtained from Acris Antibodies (Herford, Germany). Dynasore and *N*-isobutyl-*N*-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid (NNGH) were purchased from Enzo Life Science (PA, USA). Rabbit anti-human CTGF/CCN2 antibody and recombinant human CTGF/CCN2 were purchased from Peprotech (NJ, USA) and BioVendor (NC, USA), respectively.

### CELL CULTURE

Human dental pulp (fibroblast-like) cells were obtained from first premolars extracted under aseptic conditions from patients aged 20 years during orthodontic treatment. The patients gave informed consent before providing the samples. After the dental pulp had been extracted, the tissue was minced, placed on a 35-mm tissue culture dish, and covered with a sterilized glass coverslip. The explants were cultured in α-MEM supplemented with 10% FBS and antibiotics (20 U/ml penicillin G, 100 μg/ml kanamycin, 250 ng/ml fungizone) at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Once cell growth from the explants had reached confluence, the cells were detached with 0.025% trypsin in phosphate-buffered saline (PBS) and subcultured in culture flasks. For the experiments, the cells from passages 5–7 were plated at $2 \times 10^5$ cells/ml medium. This study was approved by the ethics committee of Nihon University School of Dentistry at Matsudo (No. EC09-008).

### CELL MIGRATION ASSAY

Culture-insert (ibidi, Munich, Germany) was placed on 35-mm tissue culture dishes (Iwaki, Tokyo, Japan), and human dental pulp cells were seeded at $0.20 \times 10^4$ cell/well. When the cells were confluent, to inhibit the effect of cell proliferation, the cells were pretreated with 10 μg/ml mitomycin C (Calbiochem, CA, USA) for 2 h, and washed with PBS. The Culture-Insert was then removed and the cells were incubated with α-MEM containing 1% FBS in the presence or absence of 100 ng/ml MMP-3 and anti-CTGF/CCN2 antibody (0.01, 0.1, and 1 μg/ml) or recombinant human CTGF/CCN2 (10, 50, and 100 ng/ml). The neutralization activity of anti-CTGF/CCN2 antibody has been shown on the data sheet that a concentration of 6.0–9.0 μg/ml of this antibody is required for $ND_{50}$ of the biological activity of CTGF/CCN2 (5.0 μg/ml). Photographs of the migration assay were taken at 0, 12, 24, and 48 h after stimulation using an inverted phase-contrast microscope (40×, Nikon Eclipse TE300). The cell migration was quantified by calculating the cell-covered area at five

randomly chosen sites per dish using WimScratch (Wimasis, Munich, Germany). The migration rates were calculated as follows: Migration rate = cell covered area$_{24h}$ − cell covered area$_{0h}$ and were normalized against the control.

### REAL-TIME REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (REAL-TIME RT-PCR)

Human dental pulp cells were cultured in 60-mm tissue culture dishes (Corning) in α-MEM containing 10% FBS. When the cells were confluent, they were incubated in α-MEM containing 1% FBS for 24 h, and then stimulated with MMP-3. After stimulation, the cells were washed twice with PBS. Total cellular RNA was extracted using RNeasy® mini kit (Qiagen®, Hilden, Germany) in accordance with the kit protocol. The concentration of RNA was measured by spectrophotometric absorption at 260/280 nm. Real-time PCR was performed using a One Step SYBR® PrimeScript® RT-PCR kit II (Takara, Shiga, Japan) in a total reaction volume of 25 μl containing of 1 μl of RNA (equivalent to 100 ng of total RNA), 12.5 μl of 2 × One Step SYBR® RT-PCR buffer 4, 1 μl of PrimeScript® 1-step Enzyme Mix 2, 400 nM each forward and reverse primers, and 8.5 μl of Rnase-free $dH_2O$. Amplification and detection were performed in a Thermal Cycler Dice® Real Time System (Takara) under the following conditions: reverse transcription for 5 min at 42°C and 10 s at 95°C, 50 cycles of PCR reaction for denaturation at 95°C for 5 s, and annealing/extension at 60°C for 30 s. The PCR primers for CTGF/CCN2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were based on published sequences, as shown in Table I.

### SDS–PAGE AND WESTERN BLOTTING

Cell lysates were prepared with the CelLytic™ M Cell Lysis Reagent (Sigma-Aldrich, Dorset, UK) containing Complete Mini EDTA-free (Roche, Mannheim, Germany), 100 μM PMSF, 0.2 mM EGTA, and 2 mM EDTA. Protein concentrations were adjusted using the method of Bradford [1976]. Extracted proteins were boiled at 95°C for 5 min in sodium dodecyl sulfate (SDS) sample buffer. Proteins were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) in 7.5% polyacrylamide gel and were transferred to nitrocellulose membranes overnight (12.5 V). The membranes were blocked at room temperature for 50 min in Block Ace (DS Pharma Biomedical, Osaka, Japan), and then probed for 120 min with the primary antibody rabbit anti-human CTGF/CCN2 (diluted 1: 1,000). The blots were washed three times with 10% Block Ace containing 0.05% Tween 20 and were then probed for 90 min with the secondary antibody, anti-rabbit IgG HRP-linked (Cell Signaling Technology®, Inc., MA, USA, diluted 1: 2,000). Immunoreactivity was detected using an ECL plus Western blotting detection system (GE, NJ, USA). Intensities of specific protein bands were measured using Photoshop.

### ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Human dental pulp cells were cultured in 60-mm tissue culture dishes in α-MEM containing 10% FBS. When the cells were confluent, they were incubated in α-MEM containing 1% FBS for 24 h, and then stimulated with MMP-3. Cell culture media were then collected at the indicated times after MMP-3 addition. Human CTGF/CCN2 standard and cell culture media were added to the 96-well plates, and the concentration of CTGF/CCN2 in culture medium was measured using a human CTGF ELISA development kit (Peprotech) in accordance with the manufacturer's instructions. The plate was monitored with Immuno Mini NJ-2300 (Biotec, Tokyo, Japan).

### MMP-3 ACTIVITY ASSAY

MMP-3 activity was determined by using Mca-RPKPVE-Nval-WRK(Dnp)-$NH_2$ fluorogenic peptide substrate II (R&D Systems, MN, USA) [Nagase et al., 1994; Eguchi et al., 2008]. MMP-3 and NNGH were mixed with the substrate and 50 mM Tris–HCl buffer (pH 7.5) containing 5 mM $CaCl_2$ and 0.05% Triton X-100 in 96-well black plate. After incubation in indicated times at 37°C, the fluorescence units (expressed as MMP-3 activity) were monitored with excitation at 370 nm and emission at 460 nm using ARVO MX (Perkin Elmer, Tokyo, Japan).

### STATISTICAL ANALYSIS

Results are presented as means ± SE from three different donors. Statistical analysis was performed by using Excel Statistics, 2008 (SSRI, Tokyo). The data from time-course study were analyzed by two-way ANOVA, and the data from other experiments were analyzed by one-way ANOVA. Group means were compared by Tukey's multiple comparison.

## RESULTS

### MMP-3-INDUCED CELL MIGRATION AND THE INHIBITION BY ANTI-CTGF/CCN2 ANTIBODY

It has been reported that MMP-3 stimulates the migration of HUVECs in vitro [Zheng et al., 2009]. Therefore, we first investigated whether MMP-3 induces migration of human dental pulp cells. When the cells were pretreated with mitomycin C (10 μg/ml) for 2 h to inhibit their proliferation and then stimulated by the MMP-3 (100 ng/ml), migration activity of the cells were significantly accelerated at 12, 24, and 48 h after treatment (Fig. 1A,B).

We next examined the effect of anti-CTGF/CCN2 antibody on the MMP-3-induced migration of human dental pulp cells. When the cells were incubated without (Fig. 2A) or with MMP-3 (Fig. 2C–F) in the absence (Fig. 2A,C) or presence of anti-CTGF/CCN2 antibody

TABLE I. Primers for Real-Time RT-PCR

| Gene | Gene bank ID | Primer sequences | Size (bp) |
|---|---|---|---|
| CTGF/CCN2 | NM_001901.2 | F: 5′-CTTGCGAAGCTGACCTGGAA-3′<br>R: 5′-AAAGCTCAAACTTGATAGGCTTGGA-3′ | 90 |
| GAPDH | NM_002046.3 | F: 5′-GCACCGTCAAGGCTGAGAAC-3′<br>R: 5′-TGGTGAAGACGCCAGTGGA-3′ | 138 |

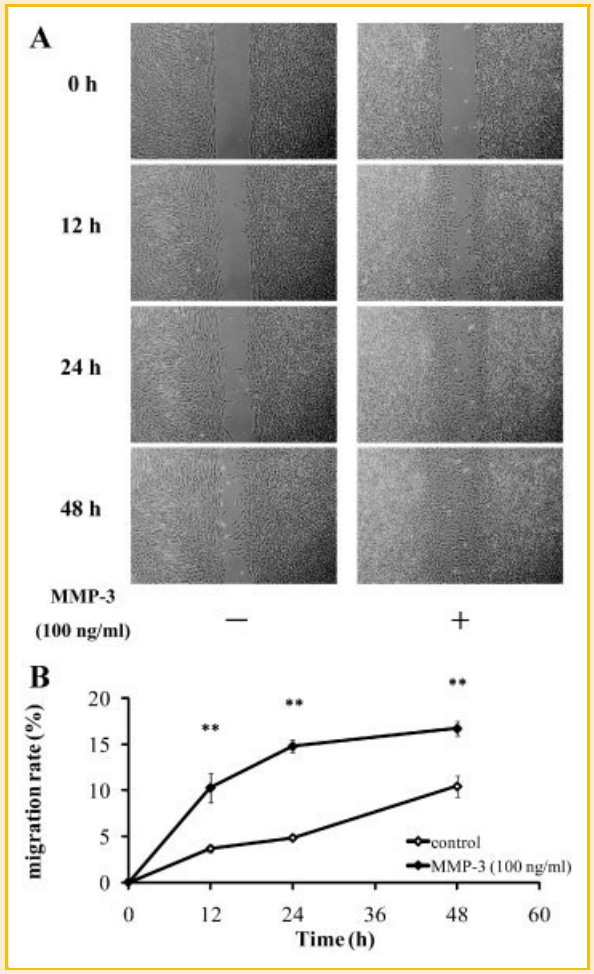


Fig. 1. Effect of MMP-3 on migration of human dental pulp cells. A: Cells were incubated with or without 100 ng/ml MMP-3 for 0, 12, 24, and 48 h. Photographs of cell migration were taken at each time using an inverted phase-contrast microscope (40×). B: The cell migration rate was quantified by calculating the cell-covered area at five randomly chosen sites per dish using WimScratch. Results are presented as mean ± SE from three independent experiments. $^{**}P < 0.01$ versus control.

(0.01–1 μg/ml, Fig. 2D–F, respectively) for 24 h, the cell migration induced by MMP-3 was significantly inhibited by the antibody in a dose-dependent manner, causing approximately a 50% inhibition at 0.1 μg/ml and a complete (100%) inhibition at 1 μg/ml. Anti-CTGF/CCN2 antibody itself had no effect on cell migration (Fig. 2B). These results suggest that CTGF/CCN2 contributes to MMP-3 induces migration of human dental pulp cells.

### CTGF/CCN2 INDUCED HUMAN DENTAL PULP CELL MIGRATION

To confirm the ability of CTGF/CCN2 on the cell migration, we also examined the effect of CTGF/CCN2 on the migration of human dental pulp cells. When the cells were incubated with CTGF/CCN2 (0, 10, 50, and 100 ng/ml), migration activity of the cells were significantly accelerated at 24 h (50 and 100 ng/ml) and 48 h (10, 50, and 100 ng/ml) after treatment (Fig. 3A,B).

### MMP-3 INDUCED CTGF/CCN2 mRNA EXPRESSION

To elucidate the relationship between MMP-3 and CTGF/CCN2, we examined the effect of MMP-3 on expression of CTGF/CCN2 mRNA in human dental pulp cells using real-time RT-PCR. When the cells were stimulated with MMP-3 (100 ng/ml), the expression of CTGF/CCN2 mRNA was significantly enhanced in a time-dependent manner, reached to a peak at 30 min and then decreased to a steady level (Fig. 4A). The mRNA expression induced by MMP-3 (0.01–1 μg/ml) for 30 min was concentration-dependent (Fig. 4B).

### MMP-3-INDUCED CTGF/CCN2 PROTEIN EXPRESSION

Next, we investigated the effect of MMP-3 on CTGF/CCN2 protein expression in human dental pulp cell lysate by Western blotting. The band cross-reacted with anti-CTGF/CCN2 antibody was detected, but which did not appear when the antibody was preabsorbed with CTGF/CCN2 protein (data not shown), indicating that CTGF/CCN2 protein expression was detected by Western blotting. The CTGF/CCN2 protein expression was significantly increased by MMP-3 (100 ng/ml) in a time-dependent manner, peaked at 1 h after stimulation and then decreased to a control level (Fig. 5A,C). The effect of MMP-3 (0.01–100 ng/ml) on the expression of CTGF/CCN2 protein was dose-dependent (Fig. 5B,D).

### MMP-3-INDUCED CTGF/CCN2 SECRETION

To confirm the secretion of CTGF/CCN2 by MMP-3 in human dental pulp cells, we determined the level of CTGF/CCN2 in culture medium using ELISA. As Figure 5E shows, the secretion of CTGF/CCN2 was stimulated by MMP-3 (100 ng/ml) in a time-dependent manner, the level peaked at 1 h after stimulation and subsequently decreased to a sustained level. The decrease of CTGF/CCN2 level appears to be caused by the protease activity of MMP-3, because CTGF/CCN2 has been reported to be cleaved by MMP-3 at the region between the VWC and TSP-1 domains [Hashimoto et al., 2002]. MMP-3 (0.01–1 ng/ml) stimulated the CTGF/CCN2 secretion in a dose-dependent manner, as Figure 5F summarizes. Taken together, it is most likely that CTGF/CCN2 produced and then secreted from the cells stimulated with MMP-3 contributes to cell migration.

### NO EFFECT OF MMP-3 INHIBITOR ON MMP-3-INDUCED CTGF/CCN2 PRODUCTION

To investigate whether the protease activity of MMP-3 is associated with MMP-3-induced CTGF/CCN2 expression, we examined effect of NNGH, a potent MMP-3 inhibitor [MacPherson et al., 1997; Alcaraz et al., 2007]. Human dental pulp cells were stimulated with MMP-3 (100 ng/ml) for 1 h in the presence of NNGH (0.013–1.3 μM), but CTGF/CCN2 protein expression induced by MMP-3 was not affected by the inhibitor (Fig. 6A,B). To confirm the protease activity of MMP-3, we also determined the MMP-3 activity by using fluorogenic substrate. The Mca-RPKPVE-Nval-WRK(Dnp)-$NH_2$ fluorogenic peptide substrate II was rapidly cleaved by MMP-3 (2.5 μg/ml), and the proteolytic activity of MMP-3 was significantly blocked by NNGH (1.3 μM) from 30 min after incubation (Fig. 6C,D). These results suggest that the protease activity of MMP-3 is not be involved in the expression of CTGF/CCN2. CTGF/CCN2 protein expression was slightly increased by NHGH in no-stimulated cells, although there is no statistical change in between control and NNGH

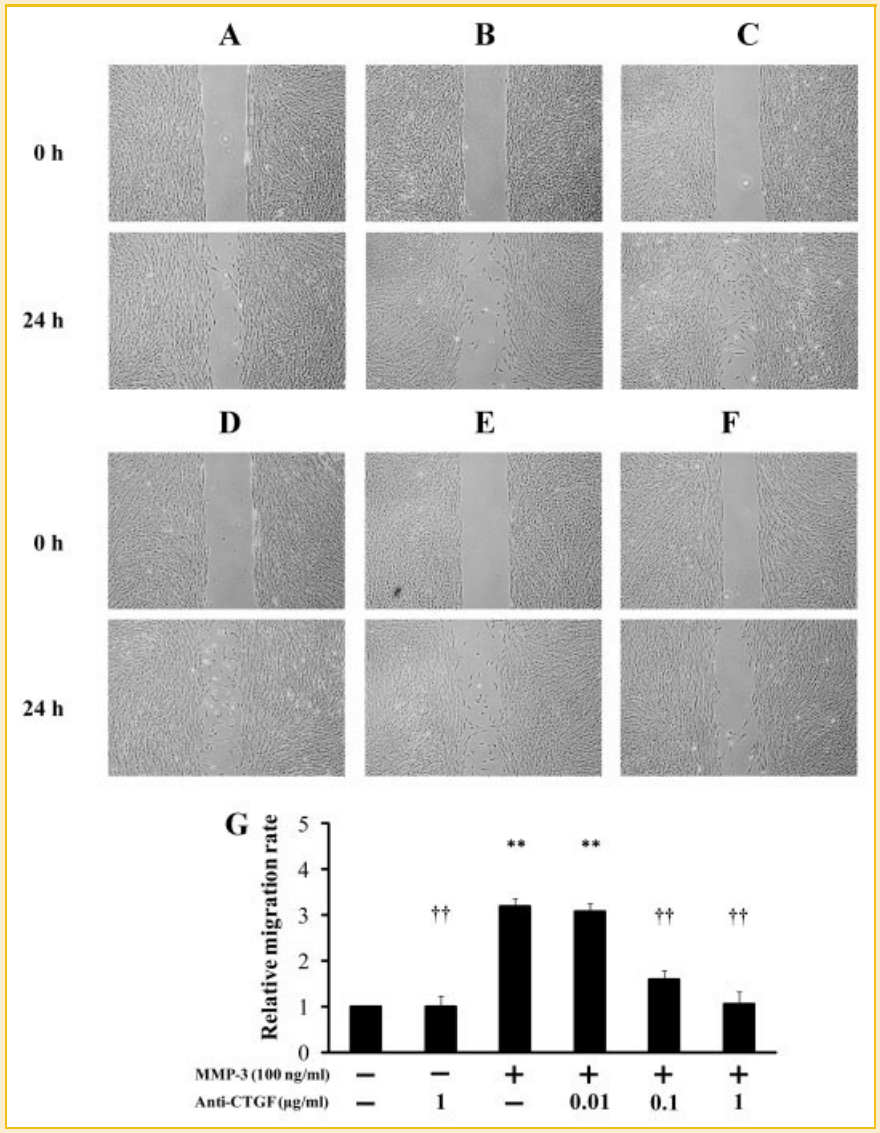


Fig. 2. Effect of anti-CTGF/CCN2 antibody on MMP-3-induced migration of human dental pulp cells. Cells were incubated without (A,B) or with 100 ng/ml MMP-3 (C–F) in the absence (A,C) or presence of 0.01 (D), 0.1 (E) and 1 μg/ml anti-CTGF/CCN2 antibody (B,F) for 0 h (upper) and 24 h (lower). Photographs of cell migration were taken using an inverted phase-contrast microscope (40×). The cell migration rate was quantified by calculating the cell-covered area at five randomly chosen sites per dish using WimScratch, and was normalized against the control (G). Results are presented as mean ± SE from three different donors. $^{**}P < 0.01$ versus control, $^{\dagger\dagger}P < 0.01$ versus 100 ng/ml MMP-3 without anti-CTGF/CCN2 antibody.

treatment. Since the expression of CTGF/CCN2 has been reported to be induced by histone deacetylation by trichostatin A (TSA), a hydroxamic acid derivative [Chiba et al., 2004], hydroxamic acid in the structure of NNGH appears to cause the slight increase in the protein expression.

### INHIBITION OF MMP-3-INDUCED CTGF/CCN2 PROTEIN EXPRESSION BY DYNAMIN INHIBITOR

It has been reported that MMP-3 translocates into cells, because fluorescence-labeled MMP-3 is detected in the cytoplasm [Eguchi et al., 2008]. However, the mechanism is unclear. We hypothesized that MMP-3 might be internalized by endocytosis process. Then effect of the dynamin inhibitor dynasore on MMP-3-induced CTGF/CCN2 protein expression was examined. When the cells were stimulated with MMP-3 (100 ng/ml) in the absence or presence of dynasore (100–300 μM) for 1 h, MMP-3-induced CTGF/CCN2 protein expression were significantly inhibited by the inhibitor in a dose-dependent manner, the MMP-3 effect completely suppressed by 300 μM dynasore in human dental pulp cells (Fig. 7). These results strongly suggest that dynamin-dependent endocytosis process contributes to MMP-3-induced CTGF/CCN2 protein expression.

## DISCUSSION

In this study, we demonstrated that (1) MMP-3 induced cell migration, (2) MMP-3 stimulated CTGF/CCN2 mRNA and protein

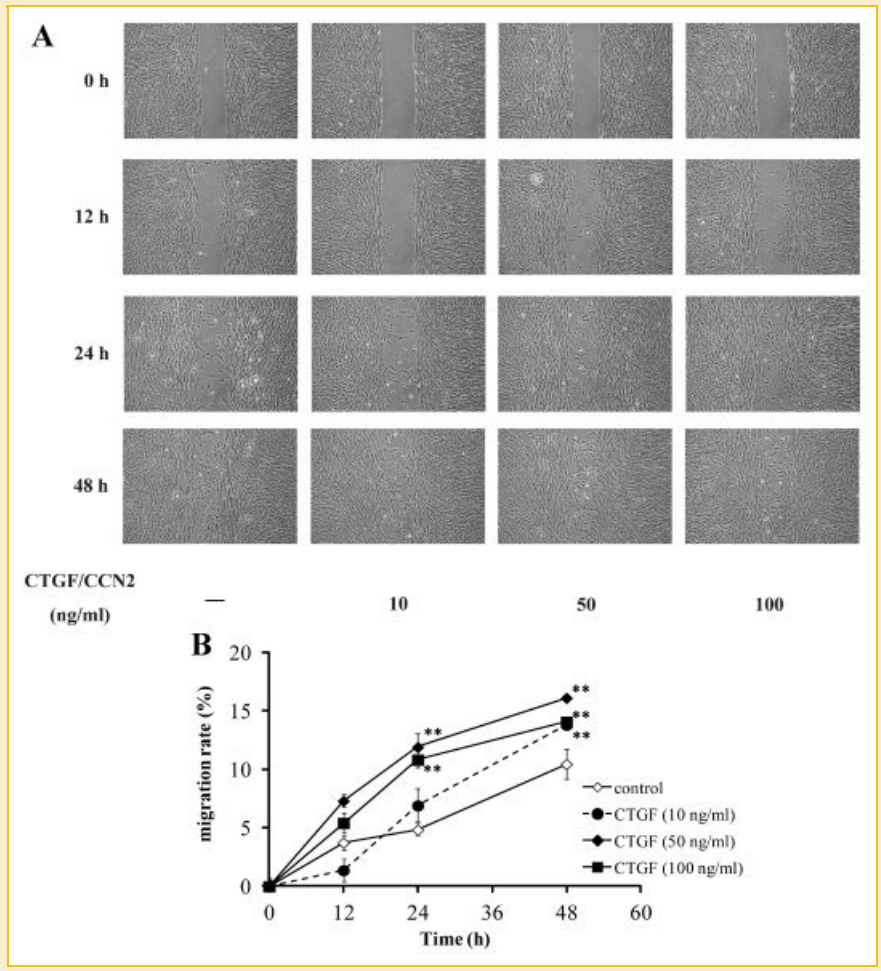


Fig. 3. Effect of CTGF/CCN2 on migration of human dental pulp cells. A: Cells were incubated with 0, 10, 50, and 100 ng/ml CTGF/CCN2 for 0, 12, 24, and 48 h. Photographs of cell migration were taken at each time using an inverted phase-contrast microscope (40×). B: The cell migration rate was quantified by calculating the cell-covered area at five randomly chosen sites per dish using WimScratch. Results are presented as mean ± SE from three independent experiments. $^{**}P < 0.01$ versus control.

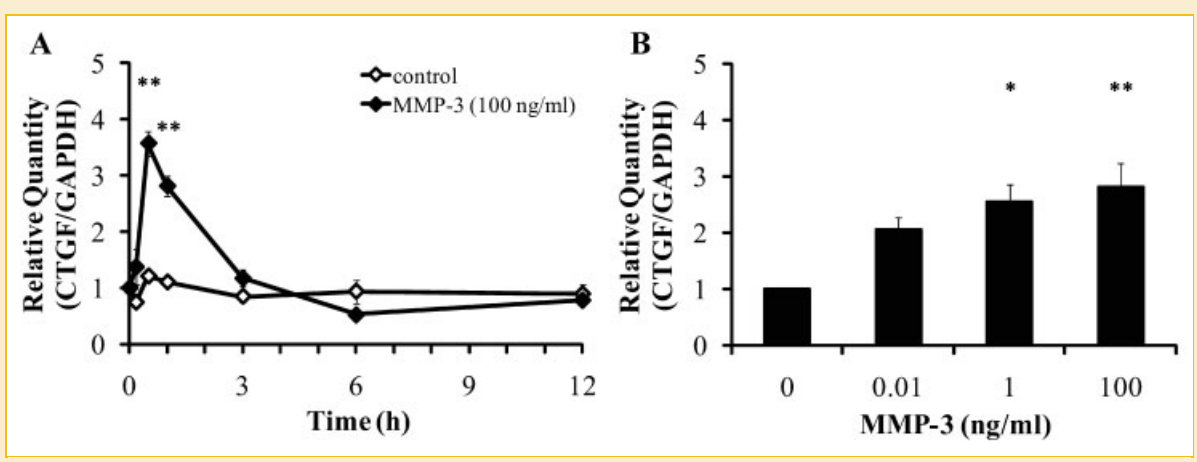


Fig. 4. Quantitative analysis of MMP-3-induced CTGF/CCN2 mRNA expression in human dental pulp cells by real-time RT-PCR. A: Cells were stimulated with MMP-3 (100 ng/ml) for the indicated times. B: Cells were stimulated with indicated concentrations of MMP-3 for 30 min. Data are calculated as the quantity of CTGF/CCN2 mRNA relative to that of GAPDH and were normalized against the control. Results are presented as means ± SE from three different donors. Statistical analysis was performed by Tukey test. $^{*}P < 0.05$; $^{**}P < 0.01$ versus control.

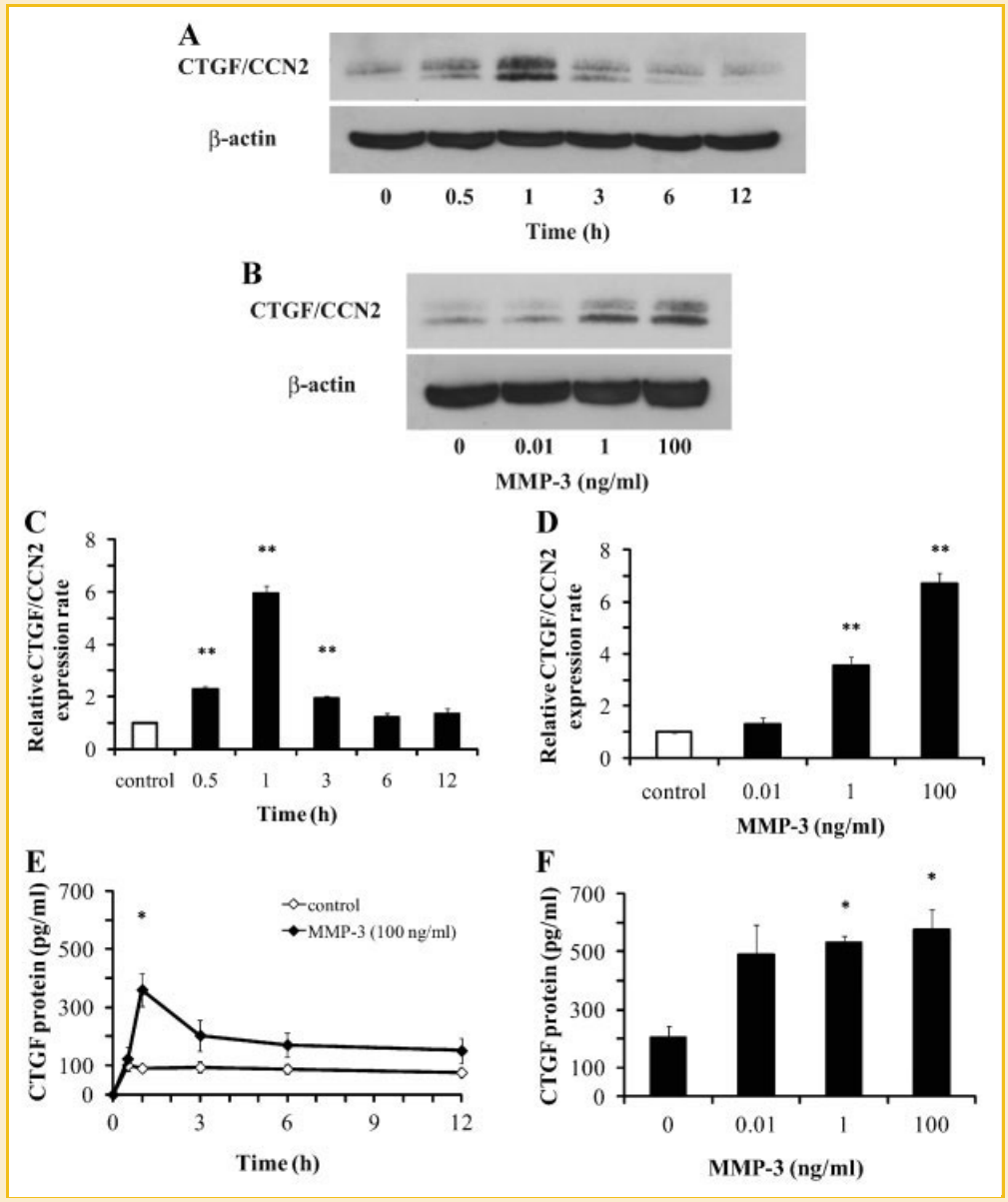


Fig. 5. A–D: MMP-3-induced CTGF/CCN2 protein expression in human dental pulp cell lysate. A: Cells were stimulated with MMP-3 (100 ng/ml) for the indicated times. B: Cells were stimulated with the indicated concentrations of MMP-3 for 1 h. CTGF/CCN2 in the cell lysate was detected by Western blotting using anti-CTGF/CCN2 antibody. C,D: Relative CTGF/CCN2 expression rate was calculated as the ratio of CTGF/CCN2 to β-actin and is normalized against control. E,F: MMP-3-induced CTGF/CCN2 protein secretion in culture medium. E: Cells were stimulated with MMP-3 (100 ng/ml) for the indicated times. F: Cells were stimulated with the indicated concentrations of MMP-3 for 1 h. Secreted CTGF/CCN2 levels were determined by ELISA. Results are presented as means ± SE from three different donors. Statistical analysis was performed by Tukey test. $^{*}P < 0.05$; $^{**}P < 0.01$ versus control.

expression and secretion, (3) CTGF/CCN2 induced cell migration, and (4) MMP-3 failed to stimulate cell migration in the presence of anti-CTGF/CCN2 antibody in human dental pulp cells. These results strongly suggest that MMP-3-induced cell migration is caused by CTGF/CCN2 newly produced and secreted by the stimulation. We also demonstrated that (1) the dynamin inhibitor dynasore inhibited MMP-3-induced CTGF/CCN2 protein expression, (2) but not the MMP-3 protease inhibitor NNGH. These observations suggest that dynamin-dependent endocytosis is involved in the effect of MMP-3 on CTGF/CCN2 expression but not the protease activity of MMP-3.

Migration of fibroblasts and their subsequent proliferation are essential for wound healing [Diegelmann and Evans, 2004]. Migration of cells such as fibroblasts, neutrophils, odontoblasts, and blood vessel cells to sites of pulp injury has been considered to be a fundamental feature of wound healing even in dental pulp. After pulp injury, dilation of blood vessels occurs, and neutrophils, one of the acute inflammatory cells, infiltrates into the site of subjacent injured pulp tissue. Following the disappearing of inflammatory cells and the emergence of fibroblast-like cells, new blood vessels are formed during dental pulp healing. In human

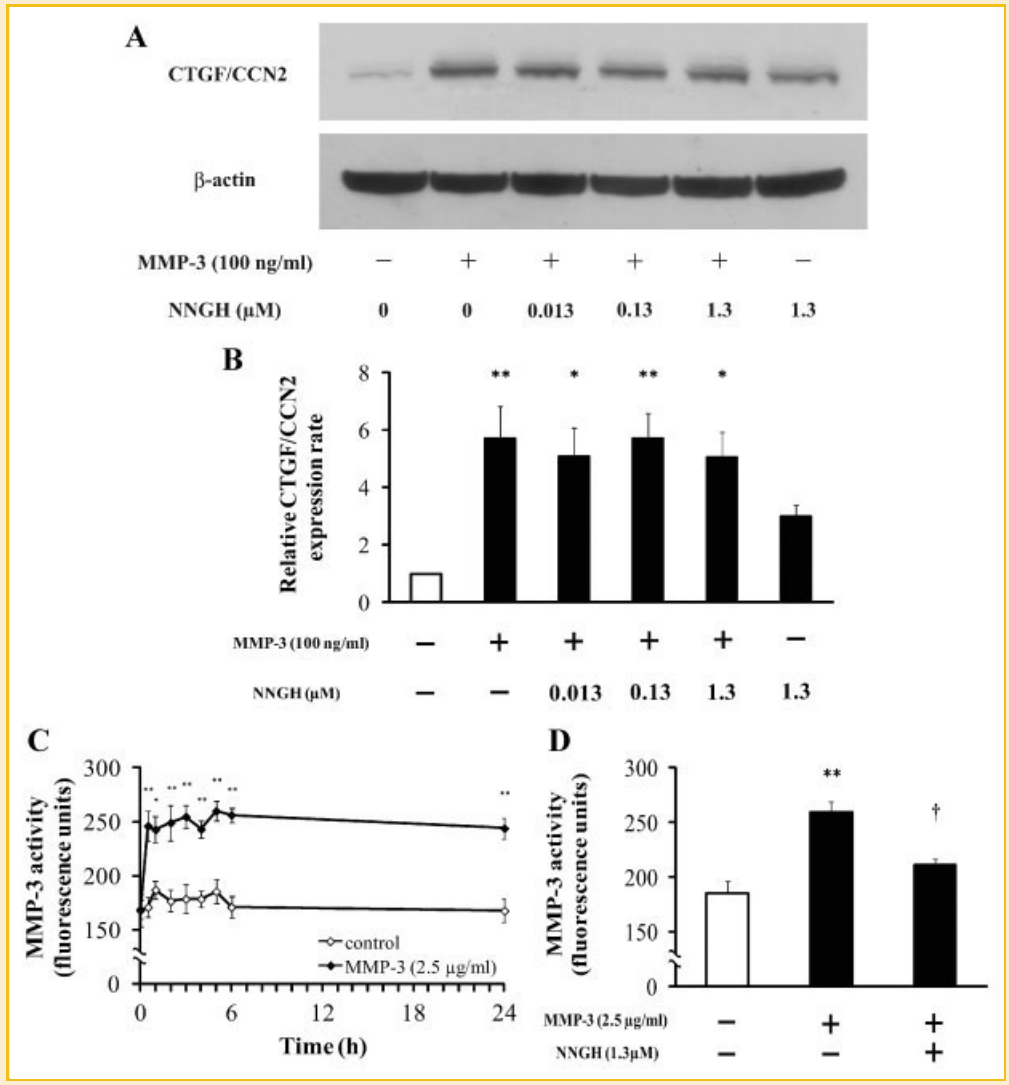


Fig. 6. No effect of NNGH on MMP-3-induced CTGF/CCN2 protein expression in human dental pulp cells. A: Cells were stimulated with 100 ng/ml MMP-3 in the absence or presence of NNGH (0.013, 0.13, and 1.3 μM) for 1 h. CTGF/CCN2 in the cell lysate was detected by Western blotting using anti-CTGF/CCN2 antibody. B: Relative CTGF/CCN2 expression rate was calculated as the ratio of CTGF/CCN2 to β-actin and is normalized against control. C: Proteolytic activity of MMP-3 (2.5 μg/ml) was confirmed by cleavage of the Mca-RPKPVE-Nval-WRK(Dnp)-NH2 fluorogenic peptide substrate II. D: Inhibitory effect of NNGH (1.3 μM) on MMP-3 activity was confirmed at 30 min after incubation. Results are presented as means ± SE from three different donors. Statistical analysis was performed by Tukey test. $^{*}P < 0.05$; $^{**}P < 0.01$ versus control. $^{\dagger}P < 0.05$ versus 2.5 μg/ml MMP-3 without 1.3 μM NNGH.

dental pulp, the level of MMP-3 has been shown to be significantly increased in acute pulpitis in comparison to asymptomatic pulp [Shin et al., 2002]. Recently, it has been demonstrated that MMP-3 promotes angiogenesis and hard tissue formation in the rat dental pulp [Zheng et al., 2009]. Previously, MMP-3 has been demonstrated to be contained in the forming enamel and dentin of the bovine molar and the rat incisor [DenBesten et al., 1989; Hall et al., 1999]. MMP-3 localized in predentin has been suggested to be participating in mineralization by regulating the turnover of proteoglycan [Hall et al., 1999]. We demonstrated here MMP-3-induced human dental pulp cell migration. These observations suggest that MMP-3 participate vital processes for healing of injured dental pulp via cell migration, angiogenesis, and formation of calcified tissue.

MMP-3 failed to induce the dental pulp cell migration in the presence of anti-CTGF/CCN2 antibody, suggesting that MMP-3 accelerates the cell migration via CTGF/CCN2. We also confirmed that CTGF/CCN2 induced the migration of human dental pulp cells. In fact, it has been reported that CTGF/CCN2 serves as an up-regulator of cell migration in HUVECs [Bradham et al., 1991; Babic et al., 1999; Shimo et al., 1999] and retinal pigment epithelium cells [Guo et al., 2009]. The antibody used in this study is made against synthetic peptide corresponding to CT domain of CTGF/CCN2. CT domain of CTGF/CCN2 has been shown to accelerate pancreatic stellate cell migration [Gao and Brigstock, 2006]. Our results supported that CTGF/CCN2 promotes cell migration via the CT domain. We also demonstrated here that MMP-3 clearly induced the expression of CTGF/CCN2 mRNA and protein and secretion in

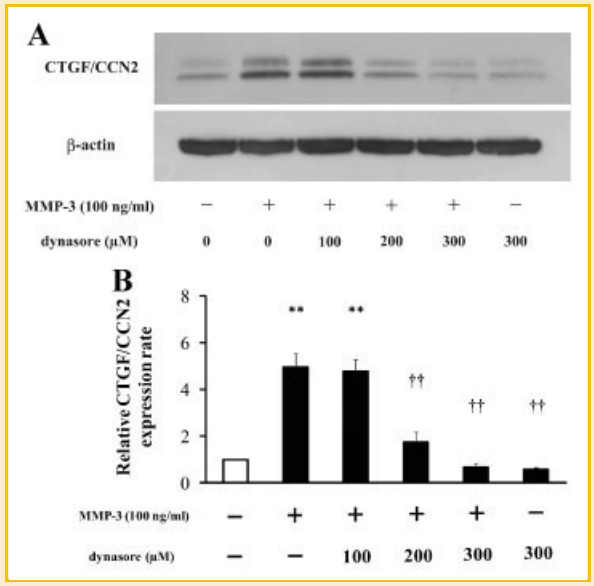


Fig. 7. Inhibition of MMP-3-induced CTGF/CCN2 protein expression by dynasore in human dental pulp cells. A: Cells were stimulated with 100 ng/ml MMP-3 in the absence or presence of dynasore (100, 200, and 300 μg/ml) for 1 h. CTGF/CCN2 in the cell lysate was detected by Western blotting using anti-CTGF/CCN2 antibody. B: Relative CTGF/CCN2 expression rate was calculated as the ratio of CTGF/CCN2 to β-actin and is normalized against control. Results are presented as means ± SE from three different donors. Statistical analysis was performed by Tukey test. $^{**}P < 0.01$ versus control, $^{\dagger\dagger}P < 0.01$ versus 100 ng/ml MMP-3 without dynasore.

human dental pulp cells. Therefore, it is conceivable that MMP-3 acts as a key factor of dental pulp wound healing through up-regulation of CTGF/CCN2.

We found dynasore clearly suppressed MMP-3-induced CTGF/CCN2 expression. Dynasore inhibits GTPase activity of the GTP-binding protein dynamin, dynamin-1 and -2 and Drp1 (mitochondrial dynamin) [Macia et al., 2006]. The role of dynamin is well characterized in the clathrin-mediated endocytosis. At the last stage of clathrin-coated vesicles formation, dynamin self-assembles to ring form at the necks of invaginated coated pits [Conner and Schmid, 2003]. Subsequently, the clathrin-coated vesicles budding occur. Because dynasore arrests endocytosis at dynamin-dependent step, our observations suggest that internalization of MMP-3 through dynamin-dependent endocytosis process contributes to MMP-3-induced CTGF/CCN2 expression in human dental pulp cells. In a human chondrocyte cell line, it has been demonstrated that MMP-3 is detected in the cell nucleus and binds with enhancer sequences in the CTGF/CCN2 promoter and activates CTGF/CCN2 transcription [Si-Tayeb et al., 2006; Eguchi et al., 2008]. Therefore, such internalization of MMP-3 appears to be important for transport the intact MMP-3 to nucleus.

Transforming growth factor-β (TGF-β) is involved in regulation of CTGF/CCN2 expression in mouse AKR-2B cells [Brunner et al., 1991] and human skin fibroblasts [Igarashi et al., 1993]. On the other hand, MMP-3 has been demonstrated to stimulate release of TGF-β1 in chondrocyte-derived ECM [Maeda et al., 2001, 2002]. Therefore, TGF-β appears to be involved in MMP-3-induced CTGF/CCN2 expression and secretion in human dental pulp cells. Regarding the mechanism underlying the MMP-3-dependent CTGF/CCN2 expression, we hypothesize that there are dual regulatory pathways, one is directly regulated by MMP-3 and another is indirectly through activating TGF-β, although we need further studies.

MMPs are zinc-dependent endopeptidases. NNGH is a derivative of hydroxamic acid, an effective zinc binding group, known as potent inhibitor of MMP-3 [MacPherson et al., 1997; Alcaraz et al., 2007]. We examined the effect of NNGH on MMP-3-induced CTGF/CCN2 expression, but NNGH failed to inhibit CTGF/CCN2 expression, although NNGH slightly induced CTGF/CCN2 protein expression. We also confirmed the activity of MMP-3 was significantly inhibited by NNGH. Therefore, it is unlikely that the protease activity of MMP-3 is involved in CTGF/CCN2 expression in human dental pulp cells.

CTGF/CCN2 is expressed during embryonic development [Surveyor and Brigstock, 1999], and its expression is minimal in normal mature tissue, but increased during the process of wound healing [Nakata et al., 2002]. It has been reported that CTGF/CCN2 null mice indicates pulmonary hypoplasia with reduced cell proliferation [Baguma-Nibasheka and Kablar, 2008]. In tooth germs, CTGF/CCN2 is also expressed in the dental lamina, inner dental epithelium, outer dental epithelium, enamel knot, preameloblasts, and mesenchyme, thus playing a role in tooth development [Shimo et al., 2002]. Expression of CTGF/CCN2 has also been confirmed in mouse periodontal ligament cells and related to proliferation and differentiation [Asano et al., 2005]. Taken together, our hypothesis is that MMP-3 in damaged dental pulp may deliver paracrine signals to undamaged neighbor dental pulp cells, and secreted-CTGF/CCN2 which is induced by MMP-3 may provide migratory and angiogenic cues to dental pulp cells and undifferentiated pulp cells to injury site. On the other side, CTGF/CCN2 has been demonstrated to mediate cell migration and adhesion through integrin $\alpha_v\beta_3$ in human dermal microvascular endothelial cells, and promotes angiogenesis in rat corneas [Babic et al., 1999]. CTGF/CCN2 has also been reported to increase the intracellular $Ca^{2+}$ concentration and enhance cell migration [Guo et al., 2009]. The study on precise role and mechanism of CTGF/CCN2 in wound healing in dental pulp is underway in our laboratory.

In conclusion, MMP-3 stimulates cell migration via CTGF/CCN2 expression and secretion dependently on dynamin-related endocytosis and independently of protease activity in human dental pulp cells. This probably takes a critical part in dental pulp wound healing.

## ACKNOWLEDGMENTS

This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) (#20592239, #22791846) and Strategic Research Base Development Program for Private Universities from The Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), 2011-2013 (S1001024).

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