

Original

## Role of CTGF/CCN2 in reparative dentinogenesis in human dental pulp

Koichiro Muromachi<sup>1)</sup>, Naoto Kamio<sup>1)</sup>, Takashi Matsumoto<sup>2)</sup>  
and Kiyoshi Matsushima<sup>1,3)</sup>

<sup>1)</sup>Department of Endodontics, Nihon University School of Dentistry at Matsudo, Matsudo, Japan

<sup>2)</sup>Department of Diagnostic Pathology, Nihon University Hospital, Matsudo, Japan

<sup>3)</sup>Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo, Matsudo, Japan

(Received 28 November and accepted 21 December 2011)

**Abstract:** Connective tissue growth factor/CCN family 2 (CTGF/CCN2) has been considered to participate in tooth development. To date, the expression and role of CTGF/CCN2 in reparative dentinogenesis have been unclear. Our previous study revealed that matrix metalloproteinase-3 (MMP-3) stimulates cell migration via CTGF/CCN2 expression and secretion in human dental pulp cells, and that this is dependent on dynamin-related endocytosis and independent of protease activity. The objective of the present study was to determine the expression of CTGF/CCN2 in reparative dentin in human carious teeth and to examine the effect of CTGF/CCN2 on mineralization in cultured human dental pulp cells. Minimal expression of CTGF/CCN2 was evident in odontoblasts subjacent to the dentin-pulp junction in healthy teeth, whereas strong expression was detected in odontoblast-like cells lining the reparative dentin subjacent to dental caries. In human dental pulp cells, CTGF/CCN2 promoted mineralization but failed to induce proliferation, suggesting that this molecule has the ability to induce the differentiation of human dental pulp cells. Taken together, the data suggest that CTGF/CCN2 is likely involved in reparative dentinogenesis through formation of hard tissue in human carious teeth. (*J Oral Sci* 54, 47-54, 2012)

**Keywords:** CTGF/CCN2; CCN family; dental pulp; reparative dentinogenesis.

### Introduction

Dentin and dental pulp constitute a functional unit referred to as the dentin-pulp complex (1). The dental pulp is enclosed by rigid dentin, which is synthesized by odontoblasts throughout life. Odontoblasts are highly differentiated cells that produce a matrix comprising collagenous and non-collagenous proteins, which are capable of mineralization. While secondary dentin is formed in response to normal physiological stimuli, the dentin-pulp complex forms tertiary dentin to protect the pulp from dental caries, tooth wear, attrition, and trauma (2). Reactionary dentin, one of the types of tertiary dentin, shows morphological similarities to primary and secondary dentin. Reparative dentin, another type of tertiary dentin, forms an irregular and less tubular structure, and often includes odontoblast-like cells that are similar to bone osteocytes. The differences between these two types of tertiary dentin appear to depend on the extent of damage. In reparative dentinogenesis, the original odontoblasts are disrupted by severe damage, and some growth factors may provoke the differentiation of pulp cells into new odontoblast-like cells (2).

Connective tissue growth factor/CCN family 2 (CTGF/CCN2) is a secretory protein belonging to the CCN family, which also includes cysteine-rich 61 (CYR61/CCN1), nephroblastoma overexpressed (NOV/CCN3), as well as Wnt-induced secreted protein-1 (WISP-1/CCN4), WISP-2 (CCN5), and WISP-3 (CCN6) (3-5).

Correspondence to Dr. Koichiro Muromachi, Department of Endodontics, Nihon University School of Dentistry at Matsudo, 2-870-1 Sakaecho-nishi, Matsudo, Chiba 271-8587, Japan  
Tel: +81-47-360-9369  
Fax: +81-47-360-9370  
E-mail: mako08014@g.nihon-u.ac.jp

The CCN proteins have four functional domains: an insulin-like growth factor binding protein-like module (IGFBP), a von Willebrand factor type C repeat module (VWC), a thrombospondin type-1 repeat module (TSP-1), and a C-terminal cysteine knot-containing module (CT), although CCN5 lacks the CT module (3-5). Our previous study demonstrated that matrix metalloproteinase-3 (MMP-3) stimulates cell migration via CTGF/CCN2 expression and secretion in human dental pulp cells, being dependent on dynamin-related endocytosis and independent of protease activity (6). MMP-3, also termed stromelysin-1, is a well known protease that can degrade collagens (types III, IV, V and IX), gelatin, aggrecan, versican, perlecan, decorin, proteoglycan, fibronectin, laminin and osteonectin (7). In human dental pulp, the level of MMP-3 has been shown to be significantly increased in acute pulpitis in comparison to asymptomatic pulp (8). Recently, MMP-3 has been shown to stimulate the migration of human umbilical vein endothelial cells (HUVECs), and to accelerate hard tissue formation and angiogenesis in rat dental pulp (9). Therefore, we hypothesized that MMP-3-induced CTGF/CCN2 could enhance wound healing in dental pulp through the formation of reparative dentin. In fact, during tooth development, CTGF/CCN2 is expressed in the dental lamina, inner dental epithelium, outer dental epithelium, enamel knot, preameloblasts, and mesenchyme, thus playing a critical role in tooth growth (10). However, details of the expression and role of CTGF/CCN2 in the mature dentin-pulp complex are still unclear.

In the present study, we investigated the localization of CTGF/CCN2 in reparative dentinogenesis in carious human teeth and the effects of CTGF/CCN2 on mineralization and proliferation of human dental pulp cells *in vitro*.

## Materials and Methods

### Materials

Fetal bovine serum (FBS),  $\alpha$ -minimal essential medium ( $\alpha$ -MEM), fungizone and trypsin were purchased from GIBCO BRL Life Technologies (Tokyo, Japan). Penicillin G and kanamycin were purchased from Meiji Seika (Tokyo, Japan). Recombinant human CTGF/CCN2 was purchased from BioVendor (Candler, NC, USA).

### Tissue preparation

Five mature third molars (healthy) and 5 carious third molars were used for this study. The patients concerned gave informed consent before providing the samples. After extraction, the crowns were sectioned and were fixed immediately with 4% paraformaldehyde for 3

days. The samples were then demineralized with 0.5 M EDTA for 4 weeks. The demineralized tissues were then processed through a graded ethanol series and xylene, and embedded in paraffin. Sections 4  $\mu$ m thick were cut on a microtome and mounted on glass slides. The paraffin sections were then deparaffinized and rehydrated. Subsequently, the sections were processed for hematoxylin and eosin (HE) staining and immunohistochemistry.

### Immunohistochemistry

The sections were boiled for 10 min in 10 mM citrate buffer (pH 6) for antigen activation and then incubated with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 min to remove endogenous peroxidase. After washing with 0.1% PBST, the sections were probed for 12 h in a moist chamber with mouse anti-human CTGF/CCN2 antibody (R&D systems, MN, USA, diluted 1:100) at room temperature. The primary antibody was revealed using the Dako ChemMate™ EnVision™ system (Dako, Tokyo, Japan), and finally the sections were visualized using 0.12 mg/ml 3,3'-diaminobenzidine (DAB; Merck, Darmstadt, Germany) containing 0.03% H<sub>2</sub>O<sub>2</sub> and counterstained with Mayer's hematoxylin. Negative controls were treated without the primary antibody. This study was approved by the ethics committee of Nihon University School of Dentistry at Matsudo (No. EC09-008).

### Cell culture

Human dental pulp cells were obtained from first premolars extracted under aseptic conditions from patients aged 20 years during orthodontic treatment. 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  $\alpha$ -MEM supplemented with 10% FBS and antibiotics (20 U/ml penicillin G, 100  $\mu$ g/ml kanamycin, 250 ng/ml fungizone) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> 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, cells from passages 5-7 were plated at  $2 \times 10^5$  cells/ml medium.

### Alizarin red S staining

Human dental pulp cells were subcultured in 35-mm tissue culture dishes in  $\alpha$ -MEM containing 10% FBS. When the cells were confluent, they were stimulated with experimental medium containing  $\alpha$ -MEM supplemented with 10% FBS, 50  $\mu$ g/ml L-ascorbic acid 2-phosphate, 2 mM  $\beta$ -glycerophosphate, and recombinant human CTGF/

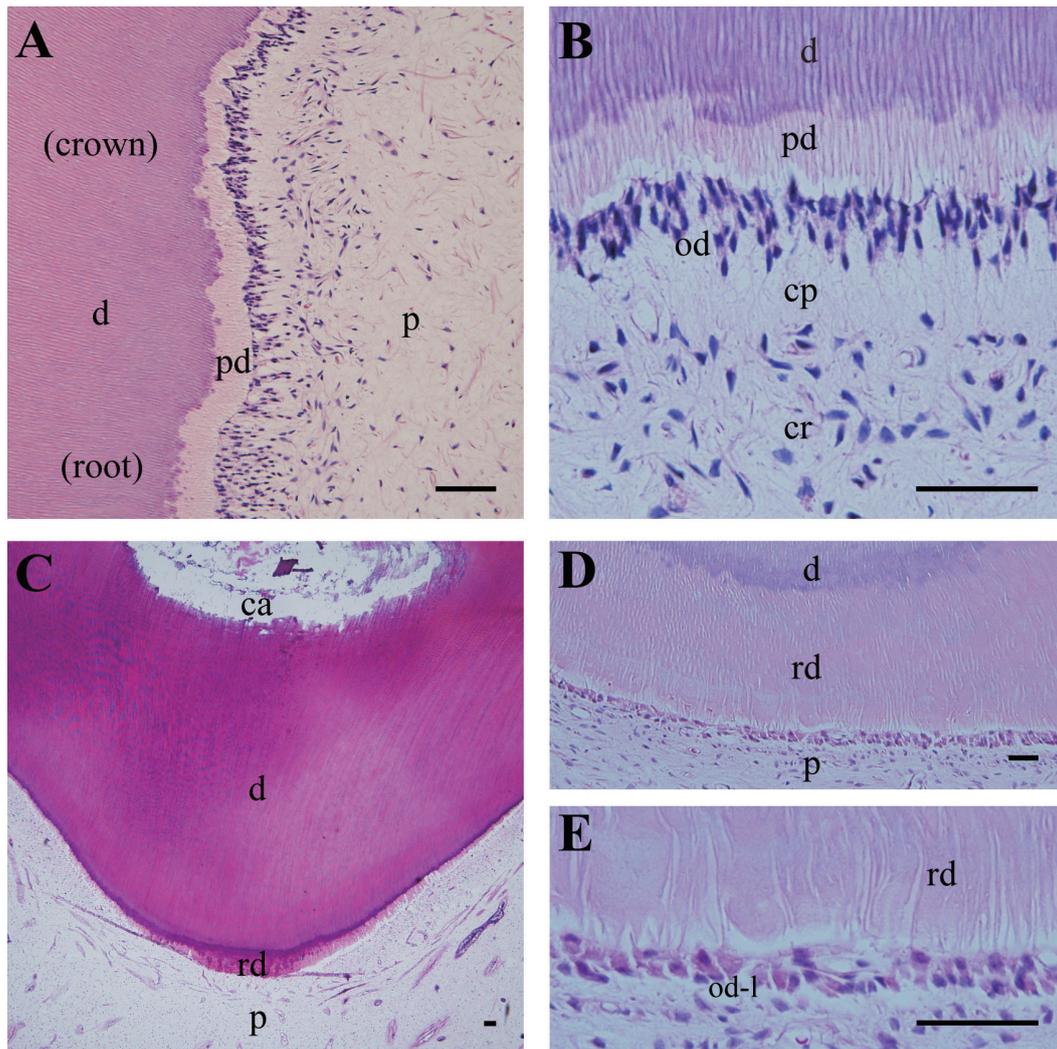


Fig. 1 HE staining of healthy and carious human teeth. (A) HE-stained section of a healthy human tooth. The cell-rich zone is more conspicuous in the coronal pulp than in the radicular pulp. (B) Healthy teeth show spindle-shaped odontoblasts aligned along the unmineralized predentin. (C) HE-stained section of a carious human tooth shows reparative dentin underlying dental caries. (D) Higher-magnification view of (C). Reparative dentin shows a less tubular form and is discontinuous with the tubules of secondary dentin. (E) Higher-magnification view of (D). Odontoblast-like cells lining the reparative dentin show a cuboidal or short columnar shape and eosinophilic cell bodies. ca: caries, cp: cell-poor zone, cr: cell-rich zone, d: dentin, od: odontoblasts, od-l: odontoblast-like cells, p: pulp, pd: predentin, rd: reparative dentin. Scale bar = 100  $\mu$ m.

CCN2 (10, 50 and 100 ng/ml) for 14, 21, or 28 days. The cultures were then fixed in 4% paraformaldehyde in PBS for 15 min. The fixed cells were incubated with 1% alizarin red S solution for 5 min, and washed three times with distilled water. For quantification of alizarin red S staining, the absorbance was measured using the method described by Gregory et al. (11).

### Proliferation assay

Human dental pulp cells were seeded in 96-well plates at  $2.5 \times 10^3$  cells/well. The cells were incubated in  $\alpha$ -MEM containing 1% FBS for 24 h, and then stimulated with each concentration of CTGF/CCN2. The number of cells

was determined using a Cell Counting Kit-8 (Dojindo, Tokyo, Japan) at 0, 12, 24, 48, and 72 h after stimulation. The kit solution was added to each well, and cultures were incubated for 2 h. Afterward, the absorbance was measured at 450 nm using an Immuno Mini NJ-2300 instrument (Biotec, Tokyo, Japan).

### Statistical analyses

The results are presented as the means  $\pm$  SE from 3 different donors. Statistical analyses were performed using the Excel Statistics software program, 2008 (SSRI, Tokyo, Japan). The data from the time-course study were analyzed by two-way ANOVA, and the data from other

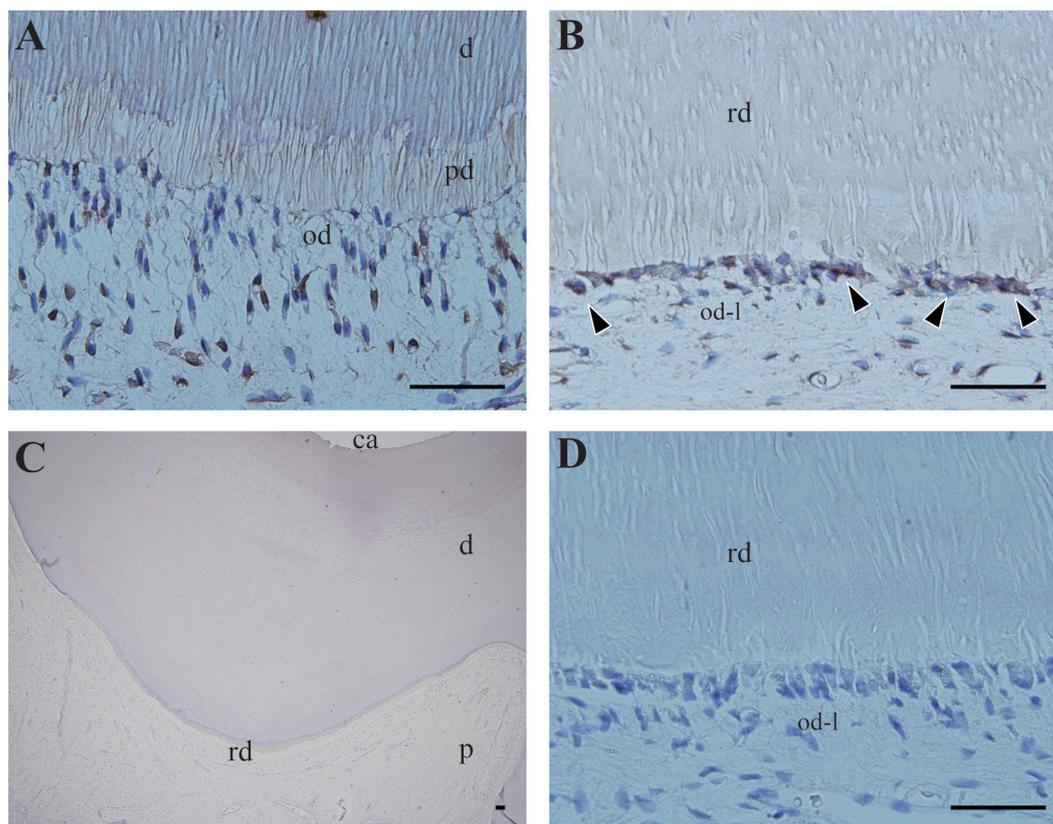


Fig. 2 Immunohistochemical localization of CTGF/CCN2 in healthy and carious human teeth. (A) In healthy teeth, the signal of CTGF/CCN2 is barely detectable in odontoblasts subjacent to the predentin. (B) Odontoblast-like cells aligned along the dentin-pulp junction subjacent to the caries lesion show a strong signal for CTGF/CCN2 (arrowheads). (C) Section of a carious human tooth shows reparative dentin underlying dental caries. (D) Section from a negative control shows no specific immunoreaction. ca: caries, d: dentin, od: odontoblasts, od-l: odontoblast-like cells, p: pulp, pd: predentin, rd: reparative dentin. Scale bar = 100  $\mu$ m.

experiments were analyzed by one-way ANOVA. Group means were compared by Tukey's multiple comparison test.

## Results

### Histological features of healthy and carious human teeth

The healthy human teeth showed an odontoblast layer, a cell-poor zone, and a cell-rich zone subjacent to the unmineralized predentin, in addition to the tubular dentin (Figs. 1A, B). The cell-rich zone was more conspicuous in the coronal pulp than in the radicular pulp (Fig. 1A). Odontoblasts subjacent to the predentin showed a spindle-shaped morphology, and were aligned with the processes of the odontoblasts oriented to the dentin-enamel junction (Fig. 1B). The carious human teeth showed irregular, less tubular forms of reparative dentin subjacent to the carious lesion (Figs. 1C-E). Dentinal tubules of reparative dentin were discontinuous with the tubules of the

secondary dentin. The cell-poor zone and cell-rich zone had disappeared. Odontoblast-like cells subjacent to the reparative dentin showed a cuboidal or short columnar shape and eosinophilic cytoplasm (Fig. 1E).

### Localization of CTGF/CCN2 in carious human teeth

We examined the localization of CTGF/CCN2 in healthy and carious human teeth using immunohistochemistry. In healthy human teeth, expression of CTGF/CCN2 was barely detected in odontoblasts subjacent to the predentin (Fig. 2A). In carious human teeth, a strong signal for CTGF/CCN2 was detected in the odontoblast-like cells located at the dentin-pulp junction subjacent to the reparative dentin (Fig. 2B). Figure 2C shows the reparative dentin subjacent to the dental caries. Sections from the negative control did not show any specific immunoreaction (Fig. 2D).

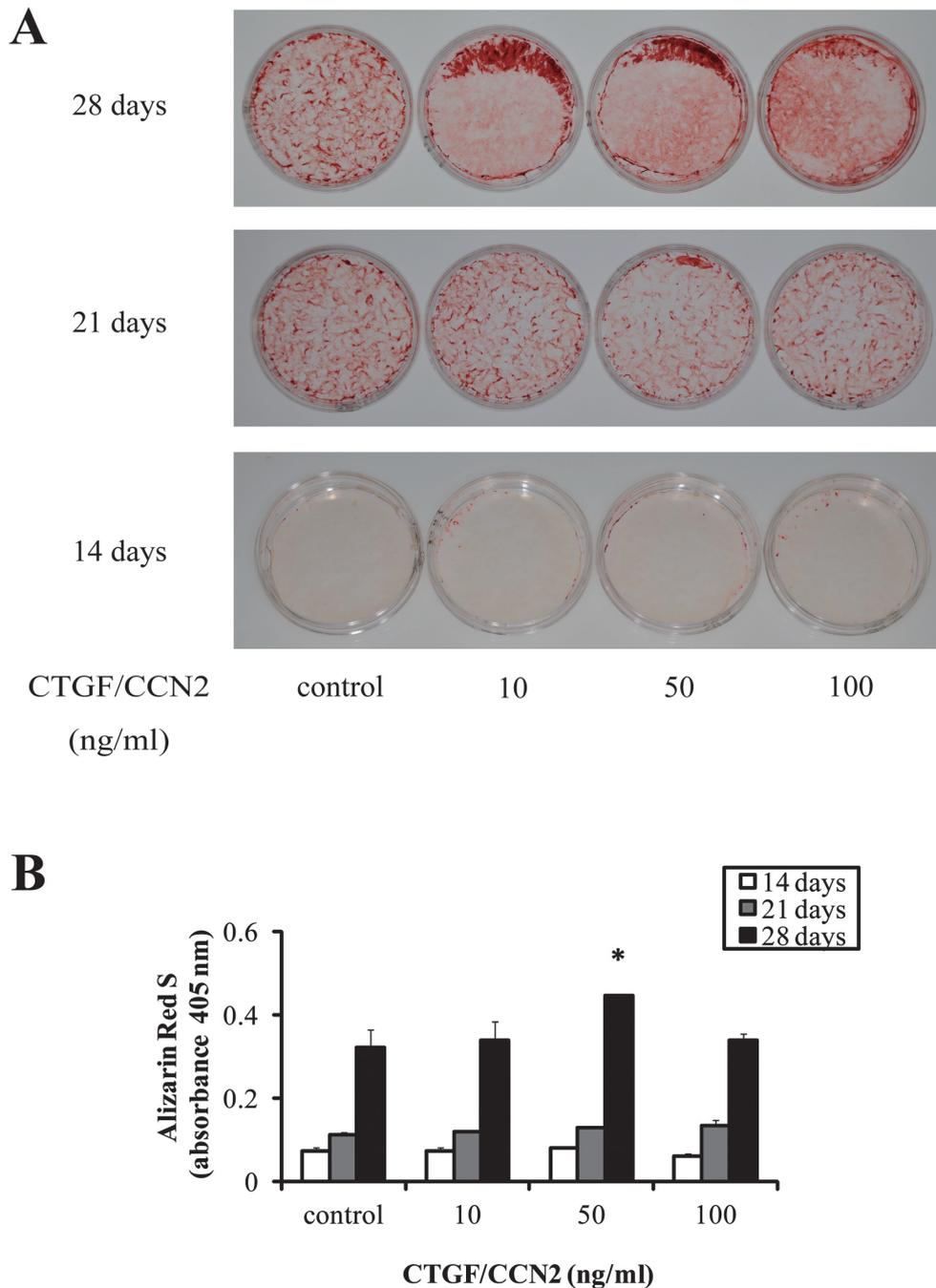


Fig. 3 CTGF/CCN2-induced mineralization of human dental pulp cells. (A) Photographs of mineralization of human dental pulp cell cultures after 14, 21, and 28 days of incubation without (control) and with CTGF/CCN2. (B) Quantification of mineralized products of human dental pulp cell cultures. Absorbance of released alizarin red S was measured at 405 nm using a spectrophotometer. Results are presented as means  $\pm$  SE from 3 different donors. Statistical analysis was performed by Tukey's test. \* $P < 0.05$  vs. control.

### CTGF/CCN2-enhanced mineralization of human dental pulp cells

Using alizarin red S staining, we investigated whether CTGF/CCN2 induces mineralization of human dental pulp cells. When the cells were stimulated with CTGF/

CCN2 (50 ng/ml), mineralization of the dental pulp cells was significantly accelerated in comparison to the control (without CTGF/CCN2) dishes at 28 days after treatment (Figs. 3A, B). Up to 21 days, CTGF/CCN2 had no significant effect on mineralization of the cultures.

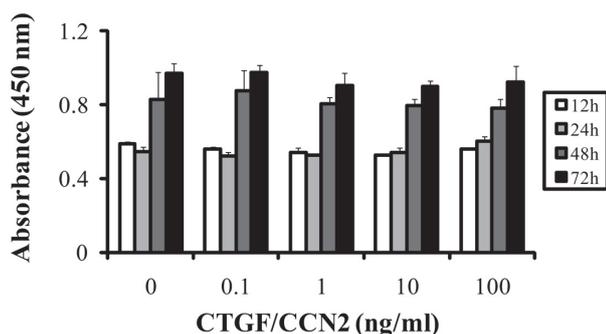


Fig. 4 Lack of effect of CTGF/CCN2 on proliferation of human dental pulp cells. Cells were stimulated with CTGF/CCN2 (0.1-100 ng/ml) for the indicated times. Results are presented as means  $\pm$  SE from 3 different donors. Statistical analysis was performed by Tukey's test.

These results suggested that CTGF/CCN2 promotes mineralization in human dental pulp cells.

#### Lack of effect of CTGF/CCN2 on proliferation of human dental pulp cells

We next investigated whether CTGF/CCN2 is associated with human dental pulp proliferation. Human dental pulp cells were stimulated with CTGF/CCN2 (0, 0.1, 1.0, 10 and 100 ng/ml) for 12, 24, 48, or 72 h, but proliferation of the cells was not significantly affected (Fig. 4). These results suggested that CTGF/CCN2 is not involved in the proliferation of human dental pulp cells.

### Discussion

In this study, we demonstrated that 1) CTGF/CCN2 is detectable in odontoblast-like cells subjacent to the reparative dentin in carious human teeth, 2) CTGF/CCN2 enhances the mineralization of human dental pulp cells, and 3) CTGF/CCN2 does not stimulate the proliferation of human dental pulp cells. These results suggest that CTGF/CCN2 is involved in reparative dentinogenesis by increasing the mineralization of human dental pulp cells.

Recent studies have shown that CTGF/CCN2 is highly expressed in immature tooth organs or stem cells from human exfoliated deciduous teeth (SHED) (10,12); however, details of its expression and role in mature dental pulp have remained unclear. Here, we demonstrated that MMP-3 induces production and secretion of CTGF/CCN2 in human dental pulp cells, subsequently inducing cell migration (6).

Strong expression of CTGF/CCN2 was detected in the cytoplasm of the odontoblast-like cells lining the reparative dentin subjacent to dental caries, relative to odontoblasts in healthy teeth. These observations suggest

that CTGF/CCN2 participates in reparative dentinogenesis by the human dentin-pulp complex. Dentin is classified into three types termed primary, secondary, and tertiary dentin in accordance with the process of its formation (13). In contrast to secondary dentin, which is formed as a reaction to normal physiological stimuli, tertiary dentin is formed in response to external stimuli such as dental caries or tooth wear. Tertiary dentin also includes the reactionary and reparative types of dentin. Reactionary dentin formed by original odontoblasts contains dentinal tubules that remain continuous with the secondary dentin, while reparative dentin formed by newly differentiated odontoblast-like cells exhibits a less tubular and osteoid appearance. It has been considered that the progenitor cells capable of differentiating into odontoblast-like cells are localized in perivascular areas, the cell-rich zone, and the inner stroma of dental pulp (14-16). These undifferentiated cells are thought to be implicated in the reparative dentinogenic response as a consequence of original odontoblast injury beneath lesions resulting from severe caries (13,17).

In odontoblasts subjacent to the dentin-pulp junction in healthy teeth, minimal expression of CTGF/CCN2 was observed. This result is in accordance with low expression of the CTGF/CCN2 gene in differentiated odontoblasts (10). Therefore it is conceivable that CTGF/CCN2 expression would be moderate in normal mature odontoblasts after tooth eruption, but would increase during the process of reparative dentinogenesis and tooth development.

Our present study also revealed that CTGF/CCN2 induced mineralization of human dental pulp cells *in vitro*. These results indicate that CTGF/CCN2 provoked by odontoblast-like cells in response to dental caries increases hard tissue formation in human dental pulp cells. CTGF/CCN2, especially the C-terminal (CT) domain, has been demonstrated to regulate Wnt signaling (18). In addition, Wnt5a regulates odontoblast differentiation during mouse tooth development (19). Therefore, in the process of formation of reparative dentin, some growth factors including CTGF/CCN2 derived from dental pulp cells such as odontoblast-like cells might trigger the mineralization of dental pulp cells. CTGF/CCN2 has also been shown to promote mineralization of various cell types such as rat osteoblasts, the mouse osteoblastic cell line MC3T3-E1, and the human osteosarcoma cell line Saos-2 (20-22).

On the other hand, CTGF/CCN2 has been considered to participate in cell proliferation (23). We also examined the effect of CTGF/CCN2 on the proliferation of mature human dental pulp cells, but found that it exerted no

significant effects. Therefore, CTGF/CCN2 appears to be involved in the differentiation, rather than the proliferation of human dental pulp cells. Taken together, the data suggest that in cases of dental caries, CTGF/CCN2 induced by odontoblast-like cells may provide paracrine signals for undifferentiated neighboring cells, thus promoting the formation of reparative dentin to protect teeth against dental caries.

In conclusion, CTGF/CCN2 was detected in odontoblast-like cells subjacent to the reparative dentin in carious human teeth. In cultured human dental pulp cells, CTGF/CCN2 enhanced mineralization but not proliferation. These results suggest that CTGF/CCN2 is involved in reparative dentin formation through mineralization in human carious teeth, thus contributing to our understanding of the mechanism underlying the repair of the dentin-pulp complex.

### Acknowledgments

This study was supported by Grants-in-Aid for Scientific Research from the JSPS (#20592239, #22791846).

### References

- Luukko K, Kettunen P, Fristad I, Berggreen E (2011) Structure and functions of the dentin-pulp complex. In: Cohen's pathways of the pulp, 10th ed, Hargreaves KM, Cohen S eds, Mosby, St Louis, 452-503.
- Tziafas D (2007) Dentinogenic potential of the dental pulp: facts and hypotheses. *Endodontic Topics* 17, 42-64.
- Holbourn KP, Acharya KR, Perbal B (2008) The CCN family of proteins: structure-function relationships. *Trends Biochem Sci* 33, 461-473.
- Moussad EE, Brigstock DR (2000) Connective tissue growth factor: what's in a name? *Mol Genet Metab* 71, 276-292.
- Lau LF, Lam SC (1999) The CCN family of angiogenic regulators: the integrin connection. *Exp Cell Res* 248, 44-57.
- Muromachi K, Kamio N, Narita T, Annen-Kamio M, Sugiya H, Matsushima K (2011) MMP-3 provokes CTGF/CCN2 production independently of protease activity and dependently on dynamin-related endocytosis, which contributes to human dental pulp cell migration. *J Cell Biochem* 10.1002/jcb.24007.
- Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T (2003) Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem* 253, 269-285.
- Shin SJ, Lee JI, Baek SH, Lim SS (2002) Tissue levels of matrix metalloproteinases in pulps and periapical lesions. *J Endod* 28, 313-315.
- Zheng L, Amano K, Iohara K, Ito M, Imabayashi K, Into T, Matsushita K, Nakamura H, Nakashima M (2009) Matrix metalloproteinase-3 accelerates wound healing following dental pulp injury. *Am J Pathol* 175, 1905-1914.
- Shimo T, Wu C, Billings PC, Piddington R, Rosenbloom J, Pacifici M, Koyama E (2002) Expression, gene regulation, and roles of Fisp12/CTGF in developing tooth germs. *Dev Dyn* 224, 267-278.
- Gregory CA, Gunn WG, Peister A, Prockop DJ (2004) An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal Biochem* 329, 77-84.
- Nakamura S, Yamada Y, Katagiri W, Sugito T, Ito K, Ueda M (2009) Stem cell proliferation pathways comparison between human exfoliated deciduous teeth and dental pulp stem cells by gene expression profile from promising dental pulp. *J Endod* 35, 1536-1542.
- Arana-Chavez VE, Massa LF (2004) Odontoblasts: the cells forming and maintaining dentine. *Int J Biochem Cell Biol* 36, 1367-1373.
- Shi S, Gronthos S (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res* 18, 696-704.
- Téclès O, Laurent P, Zygouritsas S, Burger AS, Camps J, Dejou J, About I (2005) Activation of human dental pulp progenitor/stem cells in response to odontoblast injury. *Arch Oral Biol* 50, 103-108.
- Iohara K, Zheng L, Wake H, Ito M, Nabekura J, Wakita H, Nakamura H, Into T, Matsushita K, Nakashima M (2008) A novel stem cell source for vasculogenesis in ischemia: subfraction of side population cells from dental pulp. *Stem Cells* 26, 2408-2418.
- Tziafas D, Smith AJ, Lesot H (2000) Designing new treatment strategies in vital pulp therapy. *J Dent* 28, 77-92.
- Mercurio S, Latinkic B, Itasaki N, Krumlauf R, Smith JC (2004) Connective-tissue growth factor modulates WNT signalling and interacts with the WNT receptor complex. *Development* 131, 2137-2147.
- Lin M, Li L, Liu C, Liu H, He F, Yan F, Zhang Y,

- Chen Y (2011) Wnt5a regulates growth, patterning, and odontoblast differentiation of developing mouse tooth. *Dev Dyn* 240, 432-440.
20. Safadi FF, Xu J, Smock SL, Kanaan RA, Selim AH, Odgren PR, Marks SC Jr, Owen TA, Popoff SN (2003) Expression of connective tissue growth factor in bone: its role in osteoblast proliferation and differentiation in vitro and bone formation in vivo. *J Cell Physiol* 196, 51-62.
21. Nishida T, Nakanishi T, Asano M, Shimo T, Takigawa M (2000) Effects of CTGF/Hcs24, a hypertrophic chondrocyte-specific gene product, on the proliferation and differentiation of osteoblastic cells in vitro. *J Cell Physiol* 184, 197-206.
22. Heng NH, N'Guessan PD, Kleber BM, Bernimoulin JP, Pischon N (2007) Enamel matrix derivative induces connective tissue growth factor expression in human osteoblastic cells. *J Periodontol* 78, 2369-2379.
23. Kothapalli D, Grotendorst GR (2000) CTGF modulates cell cycle progression in cAMP-arrested NRK fibroblasts. *J Cell Physiol* 182, 119-126.