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Type I Collagen Regulated Dentin Matrix Protein-1 (Dmp-1) and Osteocalcin (OCN) Gene Expression of Rat Dental Pulp Cells

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Abstract In this study, we investigated the effect of type I collagen on dentin matrix protein-1 (Dmp-1) and osteocalcin (OCN) gene expression of dental pulp cells. The mRNA level of Dmp-1 gene was down-regulated; however, OCN gene expression was up-regulated by the culture of dental pulp cells with type I collagen. These findings imply that type I collagen regulates mRNA level of Dmp-1 and OCN gene that are predominantly expressed in active odontoblasts. The change of gene expression by type I collagen was suppressed by the blocking of collagen–integrin interaction. We could conclude that the effect of type I collagen was mediated via binding of collagen to integrin receptors. J. Cell. Biochem. 88: 1112–1119, 2003.

Key words: odontoblast; gene expression; type I collagen matrix

Odontoblasts present in dental pulp play crucial roles for formation, maintenance, and repair of dentin. They synthesize an extracellular matrix that subsequently mineralizes, and forms reparative dentin [Butler, 1998]. The reparative dentin formation is enhanced by growth factors [Hu et al., 1998]. However, little is known about the mechanism of reparative dentin formation. Recently we found that type I collagen matrix accelerated mineralized tissue formation by dental pulp cells [Mizuno and Kuboki, 2000]. Collagen matrices and other extracellular matrices have been postulated to support phenotypes and tissue-specific functions in vivo [Rocha et al., 1985; Reznikoff et al., 1987], which demonstrates that extracellular matrices maintain cell functions. Type I collagen is also a major organic component of dentin, and has been shown to influence functions of osteoblasts [Andrianarivo et al., 1992; Lynch et al., 1995]. Furthermore, osteoblasts and odontoblasts share common features [Gronthos et al., 2000; Shi et al., 2001]. These findings offer the possibility that type I collagen influences the function of odontoblasts.

In this study, we investigated the effect of type I collagen on mRNA level of dentin matrix protein-1 (Dmp-1), osteocalcin (OCN), and alkaline phosphatase (ALP) of dental pulp cells, and found that Dmp-1 gene expression diminished; however, OCN gene expression was enhanced. These results indicate that type I collagen regulated gene expressions of dental pulp cells.

MATERIALS AND METHODS

Cell Culture

Rat dental pulp cells were prepared from maxillary incisors of male Wistar rats (6 weeks old). Dental pulp was removed from teeth aseptically, and incubated in phosphate buffered saline containing 0.1% collagenase at 37°C for 30 min in a CO_2 incubator. Dissociated cells were collected by centrifugation and inoculated into 25 cm^2 culture flasks. During the culture, we recognized polygonal-form and fibroblast-form cells. Then we separated them by the limited dilution method. Briefly, the cell number in the cell suspension was adjusted to 10 cells/ml, and 100 μl of cell suspension was

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put into each well of 96-well microtiter plate. After cells proliferated, both type of cells were detached by the treatment of 0.012% Pronase and 2% EDTA, and then inoculated into 25 cm^2 culture flasks, respectively. At day 10, cells reached the subconfluent stage and were detached with 0.012% Pronase and 0.02% EDTA. Then cells were inoculated into 35-mm culture dishes at the density of 1×10^5 cells/dish. When pulp cells reached to confluent stage, cells were covered by neutralized type I collagen solution, and they were maintained at 37°C for an appropriate time in a CO_2 incubator to form gel. The culture medium consisted of α modified MEM (GIBCO, NY), and 10% (v/v) fetal calf serum (GIBCO), was changed every 2 days, and continued the culture for 7days.

Measurement of ALP Activity and DNA Content

Cell-matrix layers were washed with phosphate-buffered saline to remove serum proteins, and were scraped from culture dishes. The layers were then homogenized with 400 μl of 10 mM Tris buffer (pH 7.6), and the homogenates were used for ALP activity assay [Kind and King, 1954]. One unit was estimated to be 1.4 mg of *p*-nitrophenol liberated from p-nitrophenyl phosphate in 15 min.

DNA content was determined using bisbenzimidazole (Hoechst 33258) [Labarca and Paigen, 1980]. Briefly, cell pellets were homogenized with 2 ml of 50 mM Na_2HPO_4/2.0 M NaCl (pH 7.4) and mixed with 10 μl of Hoechst 33258. The intensity of fluorescence was measured at an excitation wavelength of 356 nm and an emission wavelength of 458 nm.

Measurement of Calcium Content in the Mineralized Tissues

To estimate the formation of mineralized tissue, calcium content was measured by the OCPC method [Connerty and Briggs, 1966] after mineralized matrices were dissolved by perchloric acid under heating.

Measurement of Osteocalcin Content in the Mineralized Matrices

The mineralized collagen matrices were dissolved in formic acid and extracted osteocalcin. The osteocalcin content in the extract was measured by a commercial radioimmunoassay (BTI, IL).

Preparation of Plasmids and cDNA Templates for Riboprobe Synthesis

Templates for genes of Dmp-1, OCN, and ALP were obtained by RT-PCR using RNA extracted from dental pulp. Each PCR products were ligated into pGEM-T plasmid vector (Promega, Tokyo, Japan) for generating antisense riboprobes from the T7 promoter. Primer sequences from 5′ to 3′ for PCR were: Dmp-1 (forward; CGTTCCTCTGGGGGCTGTCC, reverse; (CCGGGATCATCGCTCTGCATC) [MacDougall et al., 1998], osteocalcin (forward; ATGAGGACCCTCTCTCTGCTC, reverse; CTAAACGGTGGTGCCATAGAT), which sequence was referred to in a previous report [Yoon et al., 1988], and ALP, which was referred from the previous report [Bonnelye et al., 2001] (forward; CCCGCATCCTTAAGGGCCAG, reverse; TAGGCGATGTCCTTG-CAGC).

Measurement of mRNA Level of Dmp-1, OCN, and ALP Gene by Northern Blotting Method

Total RNA was extracted from rat dental pulp cells using the acid guanidine-phenol-chloroform method [Chomczynski and Sacchi, 1987]. Twenty micrograms of RNA was separated by 1.4% denaturing agarose gel electrophoresis in MOPS buffer. Then RNAs were transferred onto a nylon membrane. Hybridization to RNA immobilized on the nylon membrane, was performed for 15 h at 65°C with RNA probe-labeled DIG-11-dUTP in the buffer (50% formamide, 5 × SSC, 50 mM $NaPO_4$ [pH 7.0], 7% SDS, 2% blocking buffer, 0.1% lauroylsarcosine, and 100 μg/ml of salmon sperm DNA) after prehybridization at 65°C for 1 h in the same buffer without the probe. Membranes were washed in 2 × SSC containing 0.1% SDS for 10 min at room temperature, followed by 0.1 × SSC containing 0.1% SDS for 10 min at 72°C. The detection of signals was performed by the contact of the membrane with X-P film for 5 h.

Measurement of mRNA Level by Quantitative PCR Method

The mRNA levels of Dmp-1, OCN, and ALP were measured using quantitative PCR machine (Smart Cycler System™, Cepheid, CA). The mRNAs of Dmp-1, OCN, and ALP were converted to cDNA by AMV-reverse transcriptase (Promega). The amount of cDNA was determined by quantitative PCR method. The PCR

reaction tube (25 μl) contained PCR buffer, 3 mM $MgCl_2$, 0.3 mM dNTP mixture, 0.3 μM of each primer, and Taq polymerase (1.25 unit/tube). Thermal cycling was performed at 95°C for 30 s, and 40 cycles of 95°C for 5 s, 60°C for 15 s, and 72°C for 15 s. The amount of the PCR product was estimated by the measurement of the intensity of fluorescence of Syber Green I intercalated into the PCR product. Dividing them by the amount of GAPDH mRNA normalized the mRNA levels of Dmp-1, OCN, and ALP.

RESULTS

Rat dental pulp cells are consisted of two types of cells as reported previously [Mizuno and Kuboki, 2000]. One was a polygonal-form and another was a fibroblast-form cell. The polygonal-form cells showed high ALP activity compared with that of fibroblast-form cells during the culture, and type I collagen did not influence ALP activity of polygonal-form cells (Fig. 1). In this study, we utilized polygonal-form cells as dental pulp cells.

Next we measured calcium content in the collagen matrix. As shown in Figure 2, calcium was accumulated in the collagen matrix. To confirm that high calcium content indicates mineralized tissue formation by dental pulp cells, we measured OCN content, which is a typical marker of mineralization, in the matrix, and detected OCN in the collagen matrix (Fig. 2). These results imply that type I collagen induced mineralized tissue formation by dental pulp cells, and also suggest that type I collagen influences gene expression of dental pulp cells.

Then we investigated the influence of type I collagen on Dmp-1, OCN, and ALP gene expression of dental pulp cells. These genes were verified to express in odontoblats [Guo et al., 2000; Yokose et al., 2000]. Cells were cultured with type I collagen matrix for 7 days, and their gene expressions were analyzed by Northern blotting. The mRNA level of Dmp-1 was decreased, but OCN gene expression was enhanced by the culture of dental pulp cells with type I collagen matrix (Fig. 3). On the other hand, ALP gene expression did not alter (Fig. 3), and this result was coincident to the finding that type I collagen did not influence ALP enzyme activity (Fig. 1).

To confirm the result of Northern blotting, we measured mRNA levels by quantitative PCR method. By the culture of cells with type I collagen, mRNA level of Dmp-1 and OCN gene were 1/10 and threefold compared with cells cultured in the conventional dishes, respectively (Fig. 4). On the other hand, ALP gene expression was not altered (Fig. 4).

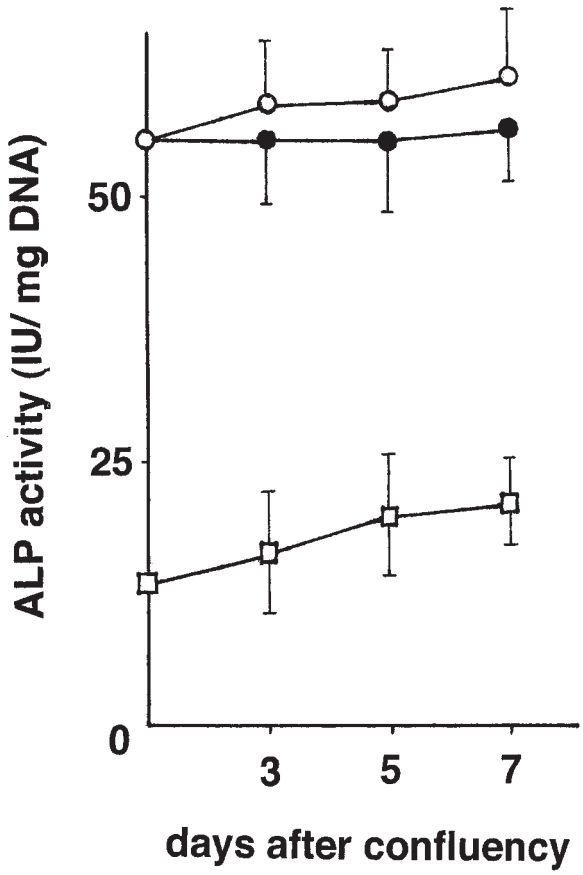

Fig. 1. ALP activity of polygonal-form and fibroblasts-form dental pulp cells after they reached the confluency. ○, Polygonal-form cells cultured in the conventional dishes; ●, polygonal-form cells cultured with type I collagen matrix; □, fibroblast-from cells in the conventional culture dishes. Data were evaluated from three samples and were shown as mean ± SD.

Type I collagen interacts with α1β1, α2β1, and α3β1 integrin receptors via DGEA amino acid sequence [Staatz et al., 1991]. In these integrins, α2β1 is a functional receptor in osteoblasts [Takeuchi et al., 1996]. To prove that the effect of collagen was mediated by the binding of collagen to α2β1 integrin receptor, we treated dental pulp cells by DGEA peptide that blocks the binding of collagen to integrin receptor competitively. Dental pulp cells were contacted with type I collagen matrix including 2 mM of DGEA peptide, and were cultured in the medium containing 2 mM of DGEA peptide

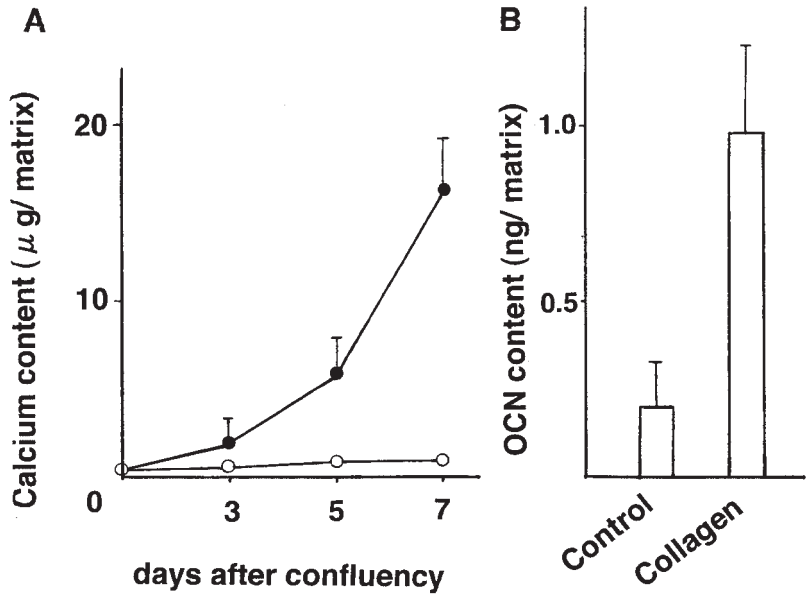

Fig. 2. **A**: Calcium content in the cell-matrix. Calcium derivatives were accumulated in the collagen matrix. ○, Cells in the conventional dishes; ●, cells cultured with type I collagen matrix. Data were evaluated from three samples, and are shown as mean ± SD. **B**: OCN content in the cell-matrix. Control, cells cultured in conventional dishes for 7 days after confluency; collagen, cells cultured with type I collagen matrix for 7 days. Matrices were dissolved in formic acid, and osteocalcin was extracted. Data were evaluated from three samples and were shown as mean ± SE.

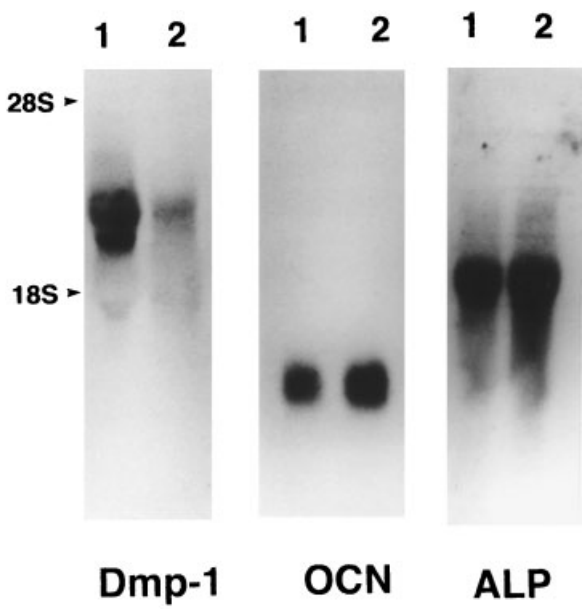

Fig. 3. Northern hybridization analysis of Dmp-1, OCN, and ALP in dental pulp cells cultured in a conventional culture dish (**lane 1**) for 7 days, and cultured with type I collagen matrix gel (**lane 2**) for 7 days. The mRNA levels of Dmp-1 decreased in the culture of cells with type I collagen matrix. On the other hand, ALP gene expression did not change.

for 7 days. By the treatment of DGEA peptide, the change of Dmp-1 and OCN gene expression by type I collagen was diminished (Table I).

Next we investigated the effect of KDGE peptide on gene expression to show that the effect of DGEA peptide is specific. KDGE peptide shares three amino acids with DGEA peptide, but does not have an activity to block the collagen–integrin interaction. When cells were treated by 2 mM of KDGE peptide, it did not interfere with the effect of collagen on dental pulp cells (Table I). These findings indicate that binding of DGEA amino acid region of collagen to α2β1 integrin receptors change the expression of Dmp-1 and OCN gene.

To verify these findings, dental pulp cells were cultured with collagen matrix containing antibody against α2 integrin subunit for 7 days to block the interaction of α2 integrin receptor with its ligand. Thirty microliters of antiserum was mixed with 1 ml of neutralized collagen solution, and the mixed solution was added on cells, and held at 37°C to polymerize. Dental pulp cells were cultured for 7 days in the α-MEM containing 10% fetal calf serum and medium was changed every 2 days. By this treatment, the change of Dmp-1 and OCN gene expression was diminished (Table I). These results imply that collagen–integrin interaction is a crucial

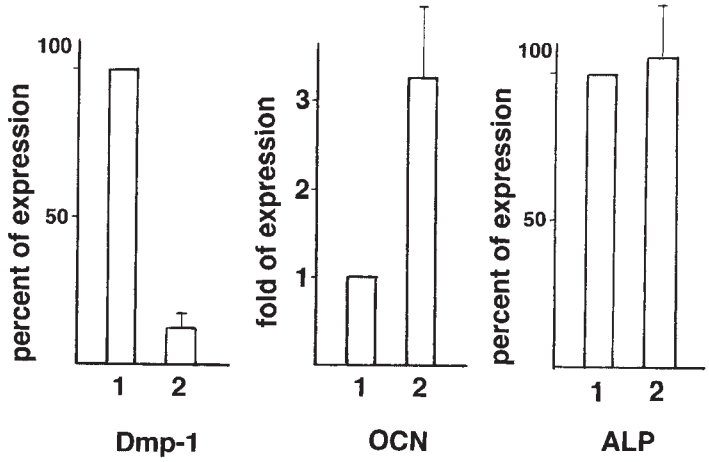

Fig. 4. Dmp-1, OCN, and ALP gene expression analyzed by quantitative PCR method. Dental pulp cells were cultured in a conventional culture dish (**lane 1**) for 7 days, or were cultured with type I collagen matrix gel (**lane 2**) for 7 days. The expression level was normalized for the level of GAPDH mRNA and was shown as percent of expression (Dmp-1 and ALP), and was shown as fold of expression (OCN).

signal for the regulation of Dmp-1 and OCN gene expression in dental pulp cells.

DISCUSSION

In this study, we demonstrated that type I collagen regulated Dmp-1 and OCN gene expression of dental pulp cells. The mRNA level of Dmp-1 was down-regulated, but OCN gene expression was up-regulated. This effect was mediated by the interaction of collagen to integrin receptors on cell membrane. Due to the influence of collagen on gene expression was diminished by the interruption of collagen–integrin interaction with DGEA peptide or anti-α2 antibody.

It has been postulated that collagen modulates several phenotypes of cells including osteoblastic differentiation [Rocha et al., 1985; Reznikoff et al., 1987; Mizuno and Kuboki, 1994]. These effects are mediated by the interaction of collagen with integrin receptors present on cell membrane [Lynch et al., 1995], and the interruption of collagen-integrin abolished the effect of collagen on cells [Takeuchi et al., 1996; Mizuno and Kuboki, 2001]. Our findings were consistent with these findings.

In our study, ALP gene expression of dental pulp cells was not modulated by the culture with type I collagen for 7 days. Lynch et al. [1995] reported that ALP gene expression of rat calvaria osteoblasts was down-regulated by the culture on collagen matrix for over two weeks. However the effect of collagen was not clear when cells were cultured for 9 days. Therefore, the effect of collagen on ALP gene expression is not definite when cells were cultured with collagen in a short time.

On the other hand, up-regulation of OCN gene expression was observed in osteoblasts cultured on collagen matrix within 9 days [Lynch et al., 1995]. These findings are consistent with our results that type I collagen induced OCN gene expression of dental pulp

TABLE I. Effect of DGEA, KDGE Peptide, and Anti-α2 Antibody on Dmp-1 and OCN Gene Expression

	Dmp-1 percent of control expression	OCN fold of control expression
Control	100	1
Collagen	12 ± 2.5	2.9 ± 0.6
Collagen + DGEA peptide	40 ± 5.9	1.8 ± 0.7
Collagen + KDGE peptide	15 ± 6.4	2.6 ± 0.5
Collagen + anti-α2 antibody	38 ± 6.2	2.1 ± 0.6

Gene expression was analyzed by quantitative PCR method. Data were evaluated from three independent experiment and are shown as mean ± SE.

cells. Thus type I collagen accelerated OCN gene expression in a short time.

OCN gene expression is regulated by the transcription factor, Cbfa-1. Cbfa-1 is expressed only in cells of the osteoblastic lineage, and the expression of Cbfa-1 in non-osteoblastic cells leads to osteoblastic specific gene expression [Ducy et al., 1997]. Furthermore, total loss of Cbfa-1 leads to inhibit osteoblastic differentiation [Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997]. These results indicate that Cbfa-1 is a critical for OCN gene expression and osteoblastic differentiation, and its function is non-redundant with other factors during development. Odontoblats are similar cells to osteoblasts in aspect of mineralized tissue formation, protein production, and reaction to growth factors [Gronthos et al., 2000; Shi et al., 2001]. Therefore the regulation of OCN gene expression by Cbfa-1 observed in osteoblasts, might be present in odontoblats.

There are two kinds of Cbfa-1 binding site: osteoblast-specific element (OSE) 1 and 2, in the promoter region of OCN gene [Frendo et al., 1998]. When Cbfa-1 binds OSE-1 that is a major *cis*-acting element, the transcription of OCN gene is enhanced [Frendo et al., 1998]. To promote the affinity of Cbfa-1 for *cis*-acting element, phosphorylation of Cbfa-1 is prerequisite, and mitogen-activated protein kinase (MAPK) plays a role for the phosphorylation of target proteins including Cbfa-1 [Xiao et al., 2000].

Collagen activates MAPK cascade by the interaction with integrin receptors of cell membrane. Integrins are considered to mediate the extracellular signal into cells via proteins; talin and vinculin [Miyamoto et al., 1995]. In cells, signal transduction molecules stimulate tyrosine phophorylation, followed by the activation of MAPK and other pathways [Lin et al., 1997]. These findings imply that up-regulation of OCN gene expression induced by type I collagen was mediated by the activation of Cbfa-1.

On the contrary, collagen suppressed Dmp-1 gene expression in this study. There is a possibility that Cbfa-1 mediates the effect of collagen, and suppresses Dmp-1 gene expression. However, the definite binding site of Cbfa-1 in Dmp-1 promoter region was not present [Ji et al., 1998; Thotakura et al., 2000]. These findings may imply that the regulatory mechanism of Dmp-1 gene expression was different from that of OCN gene expression. Our speculation is supported by the report that the expression pattern of Dmp-1 in rat developing mandibular bone is different from osteocalcin and type I collagen [Kamiya and Takagi, 2001].

OCN appears immediately before the start of mineralization [Nakashima, 1994], and is a major non-collagenous protein synthesized by osteoblasts, odontoblasts, and cementoblasts [Bronckers et al., 1989]. The expression of OCN increases as the osteoblastic differentiation proceeds [Mizuno and Kuboki, 2001]. These findings imply that OCN regulates mineralized tissue formation. However, it was reported that OCN possessed the chemotactic activity to monocytes that are precursors of osteoclast [Mundy and Poser, 1983], and bone formation was enhanced in osteocalcin-deficient mouse [Ducy et al., 1996]. Therefore OCN might play a crucial role for the regulation of mineralized tissue turnover.

Dmp-1 is an acidic phosphoprotein originally identified in dentin [George et al., 1993]. Full amino acid sequence of Dmp-1 was determined from cDNA library of rat incisors, and Dmp-1 possessed high amounts of Asp and Glu in the molecule [George et al., 1993]. Fifty-two percent of serine residues could be phosphorylated by casein kinase I and II-like kinase [George et al., 1993]. Dmp-1 has a RGD sequence in a molecule, and promotes the attachment of rat pulp cells and mouse osteoblastic cells; however, CHO cells did not show the affinity for Dmp-1 [Kulkarni et al., 2000]. These findings imply that Dmp-1 regulates the mineralization process, and influenced the activity of cells via cell-attachment.

Dmp-1 gene is expressed in odontoblasts, bone, cementum, and enamel [D'Souza et al., 1997; MacDougall et al., 1998], as well as in fetal cattle brain [Hirst et al., 1997]. Furthermore, Dmp-1 gene expression is definitely suppressed in odontoblasts that form secondary or physiological dentin [D'Souza et al., 1997]. From these observations, we hypothesized that the physiological function of collagen is to promote the activity of dentin formation by dental pulp cells.

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