J Periodont Res 2004; 39; 199–204
Printed in the UK. 



JOURNAL OF PERIODONTAL RESEARCH
doi: 10.1111/j.1600-0765.2004.00725.x

# Effect of carnosine on runt-related transcription factor-2/core binding factor α-1 and Sox9 expressions of human periodontal ligament cells

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*Ito-Kato E, Suzuki N, Maeno M, Takada T, Tanabe N, Takayama T, Ito K, Otsuka K. Effect of carnosine on runt-related transcription factor-2/core binding factor α-1 and Sox9 expressions of human periodontal ligament cells. J Periodont Res 2004; 39; 199–204. © Blackwell Munksgaard, 2004*

*Background:* Previous studies have reported that β-alanyl-L-histidine (carnosine) enhanced the process of wound healing by stimulating effusion at the initial stage of inflammation, and also enhanced runt-related transcription factor-2/core binding factor α-1 (RUNX2/Cbfa1), Sox9, bone morphogenetic protein-2 (BMP-2) and BMP-7 expressions of human periodontal ligament cells.

*Objectives:* In order to clarify the relationship between RUNX2/Cbfa1 or Sox9 expressions and BMP-2 or BMP-7 expressions enhanced by carnosine, we determined the effect of carnosine on the expression of BMP receptors and activation of their downstream signaling molecules in human periodontal ligament cells.

*Material and methods:* Human periodontal ligament cells were cultured with α-minimum essential medium containing 10% fetal bovine serum with or without $10^{-4}$ or $10^{-5}$ M carnosine for up to 10 days. The gene expression of BMP receptors, RUNX2/Cbfa1 and Sox9 was measured using semiquantitative reverse transcription-polymerase chain reaction. Phosphorylation of Smad1 was determined using a western blot analysis.

*Results:* Alkaline phosphatase activity increased in cultures with carnosine. Among the BMP receptors, expression of ActR-I and BMPR-II increased in cultures with carnosine, whereas expression of BMPR-IA, BMPR-IB, ActR-IIA and ActR-IIB was not affected. Culture with carnosine increased phosphorylation of Smad1, a signal-transducing molecule for BMP-2 and BMP-7. Noggin reduced carnosine-induced up-regulation of RUNX2/Cbfa1 and Sox9 mRNA, suggesting that BMPs were responsible for up-regulating RUNX2/Cbfa1 gene expression in human periodontal ligament cells.

*Conclusion:* These results suggest that carnosine enhance RUNX2/Cbfa1 and Sox9 expression of human periodontal ligament cells via the autocrine action of BMP-2 or BMP-7 produced by the cells.

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Key words: carnosine; human periodontal ligament cells; runt-related transcription factor-2/core binding factor α-1; Sox9

Accepted for publication November 25, 2003

One of the aims of periodontal treatment is recovery of periodontal bone defects caused by periodontal disease. Research on periodontal regeneration has generally focused on the major organic components, such as enamel matrix derivatives and growth factors, and the clinical effectiveness of these has been reported (1–4).

In recent years, the effect of dipeptide on bone metabolism has been examined *in vitro* using β-alanyl-L-histidinato zinc (AHZ), in which zinc is located in the β-alanyl-L-histidine (carnosine) (5–7). Yamaguchi *et al*. (5) reported that AHZ promotes osteogenesis via increased alkaline phosphatase activity and DNA synthesis by osteoblastic MC3T3-E1 cells. Furthermore, additional studies reported that the intracellular signal transduction of AHZ is mediated by protein kinase C (8, 9), and that AHZ promotes the differentiation of osteoblasts and suppresses the formation of osteoclasts (10). However, it is not certain whether the action of AHZ on the osteogenesis is based on carnosine or zinc.

Nagai *et al*. (11) demonstrated that carnosine stimulated the biosynthesis of nucleic acids and collagen, and enhanced the process of wound healing by stimulating effusion at the initial stage of inflammation. Maeno *et al*. (12) demonstrated that gene expression of runt-related transcription factor-2/core binding factor α-1 (RUNX2/Cbfa1), Sox9, bone morphogenetic protein-2 (BMP-2) and BMP-7 of human periodontal ligament cells was increased in cultured with carnosine. It is generally accepted that the periodontal ligament is a heterogeneous cell population, and that some of these cells are capable of differentiating into either osteoblasts or cementoblasts. Therefore, it has been hypothesized that periodontal ligament cells play a role in promoting periodontal regeneration (13).

Together, these studies suggest that treatment with carnosine may be useful in the regeneration of alveolar bone defects. This led us to investigate the effect of carnosine on the differentiation of human periodontal ligament cells. Here, we determined the effect of carnosine on the expression of BMP receptors at the message level, and determined its effect on Smad1 expression and alkaline phosphatase (ALPase) activity at the protein level. We also determined the effect of noggin on carnosine-induced up-regulation of RUNX2/Cbfa1 and Sox9 mRNA at the message level.

## Material and methods

### Cell culture

Human periodontal ligament explants were obtained from premolars or third molars that had been extracted from four healthy patients for orthodontic reasons. Informed consent was obtained prior to extraction. The study was in accordance with the Helsinki Declaration of 1975, as revised in 1983, and was also approved by the ethics committee of Nihon University School of Dentistry. The periodontal ligament tissue that was attached to the middle one-third of the root surface of the extracted teeth was scraped off with a scalpel. The tissue was cultured in α-minimum essential medium (Gibco BRL, Rockville, MD, USA) containing 10% (v/v) fetal bovine serum (HyClone Laboratories, Logan, UT, USA) and antibiotics at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$. To examine the effect of carnosine (Hamari Chemicals, Osaka, Japan) on RUNX2/Cbfa1 or Sox9 expressions, human periodontal ligament cells were inoculated onto 100-mm tissue culture plates at a density of $5 \times 10^6$ cells/cm$^2$. After an overnight incubation, the human periodontal ligament cells were cultured for up to 10 days with α-minimum essential medium containing 10% fetal bovine serum with or without $10^{-4}$ or $10^{-5}$ M carnosine.

### Determination of cell proliferation

Human periodontal ligament cells were placed into 96-well microplates at a density of $2 \times 10^4$ cells/cm$^2$ and cultured with or without carnosine for up to 10 days. At the times indicated, the medium was exchanged for fresh medium containing 10% (v/v) cell counting kit solution (Wako Fine Chemical, Osaka, Japan), and then incubated for a further 2 h. After incubation, the intensity of the reaction products was measured at 450 nm with a microtiter plate reader (Titertek Multiskan Plus, Flow Laboratories, McLean, VA, USA). The relative cell numbers (relative absorbance) were calculated from a standard curve.

### Determination of ALPase activity

Human periodontal ligament cells were placed into 96-well microplates at a density of $2 \times 10^4$ cells/cm$^2$ and cultured with or without carnosine for up to 10 days. ALPase activity was determined as described by Ohshima *et al*. (14). The amount of *p*-nitorophenol released by the enzyme reaction were measured at 405 nm using a Titertek Multiskan Plus. One unit of ALPase activity was defined as 1.0 μmol *p*-nitrophenol liberated per minute. The enzyme activity was recorded as milliunits (mU)/$10^4$ cells.

### Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Human periodontal ligament cells were cultured for up to 10 days with or without carnosine, and total RNA was isolated from the cultured cells on days 5, 7 or 10 of culture with an RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA). The amount of RNA was equalized using a human β-actin competitive PCR kit (Takara Shuzo, Shiga, Japan). The mRNA was converted into complementary DNA (cDNA) with an RNA PCR kit (GeneAmp, Perkin Elmer, Branchburg, NJ, USA). The single-stranded cDNA equivalent of 200 ng of total RNA was then subjected to PCR using gene-specific primers as shown in Table 1 (15–18). The PCR reaction was performed for 23 cycles for RUNX2/Cbfa1, for 25 cycles for BMPR-IA, BMPR-IB, ActR-I, BMPR-II, ActR-IIA and ActR-IIB, for 30 cycles for Sox9, and for 20 cycles for glyceraldehydephosphate dehydrogenase (GAPDH) in a thermal cycler (Perkin Elmer) under the following

*Table 1.* Polymerase chain reaction primers used in experiments

| Target | Forward primer | Reverse primer | bp | Reference no. |
|---|---|---|---|---|
| RUNX2/Cbfa1 | 5′-TGAGGCGGGACACCTACTCTCATA-3′ | 5′-AACCGCACCATGGTGGAGATCAT-3′ | 271 | (15) |
| Sox9 | 5′-ATCTGAAGAAGGAGAGCGAG-3′ | 5′-TCAGAAGTCTCCAGAGCTTG-3′ | 263 | (16) |
| BMPR-IA | 5′-GGGTGGGCACCAAACGCTAC-3′ | 5′-CCACTCTAATTCCACCCATGCC-3′ | 474 | (17) |
| BMPR-IB | 5′-ACTTGCTGTATTGCTGACCTGG-3′ | 5′-GGCTTTCTGCAGAGATGCTTAC-3′ | 512 | (17) |
| ActR-I | 5′-TTGCATAGCAGATTTGGGCCTG-3′ | 5′-CAGTCAGGCCAGCATTAGGTCC-3′ | 556 | (17) |
| BMPR-II | 5′-AACATTTACAGAGTGCCTTTGATG-3′ | 5′-AGCTGATTCACAGTCCCTCAAG-3′ | 468 | (17) |
| ActR-IIA | 5′-CGGGAAAATGGGAGCTGCTGC-3′ | 5′-CAATCCCCGCAATTAACATAAGTG-3′ | 467 | (17) |
| ActR-IIB | 5′-GACACGGGAGTGCATCTACTAC-3′ | 5′-GATGTCCACATGACCGTAGGG-3′ | 442 | (17) |
| GADPH | 5′-ATCACCATCTTCCAGGAG-3′ | 5′-ATGGACTGTGGTCATGAG-3′ | 318 | (18) |

RUNX2/Cbfa1, runt related transcription factor-2/core binding factor α-1; GADPH, glyceraldehydephosphate dehydrogenase.

conditions: initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The number of PCR cycles used were within the exponential phase of the amplification curve. The PCR products were loaded onto a 1.5% agarose gel, visualized with ethidium bromide, and photographed. The intensities of the PCR products were quantified on a computer using a scanner (Epson GT-9500, Seiko Epson, Tokyo, Japan) and digital image analysis software (Digital Science 1D, version 2.0, Eastman Kodak, New Haven, CT, USA).

### Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting

After stimulation in carnosine, human periodontal ligament cells were scraped and sonicated for 15 s. Samples containing equal amounts of protein were subjected to SDS–PAGE.

SDS–PAGE was carried out in 5–20% gradient cross-linked polyacrylamide gels, with a discontinuous Tris-glycine buffer system, as previously described by Laemmli (19). The electrophoresis was performed at 150 V for 60 min.

Immunotransfer analysis was performed on a semidry transfer unit with a continuous buffer system at a constant amperage of 0.8 mA/cm$^2$ for 60–90 min. On completion of the transfer, the transfer membrane was treated with 25% (v/v) blocking reagent in Tris-buffered saline at 4°C for 18 h. The sheet was washed with Tris-buffered saline containing Tween-20, and then incubated at room temperature for 90 min with anti-p-Smad1 (Santa Cruz Biotechnology, CA, USA). The sheet was washed in Tris-buffered saline containing Tween-20 and incubated at room temperature for 1 h with second antibody [rabbit anti-goat IgG (H + L) conjugated with biotin, Zymed Laboratories, San Francisco, CA, USA). The sheets were washed in Tris-buffered saline containing Tween-20, and then incubated for 30 min at room temperature with horseradish peroxidase-conjugated streptavidin. Immunoreactive proteins were visualized by producing chemiluminescence using a commercial kit (Amersham Lifescience, Buckinghamshire, UK), and exposure of the blots to X-ray film (Eastman Kodak). As a control, membranes were exposed to normal rabbit serum; the serum concentration was adjusted to the same dilution as the anti-p-Smad1 used.

### Noggin binding assay

Human periodontal ligament cells were placed into 60-mm tissue culture plates at a density of $7 \times 10^3$ cells/cm$^2$ and cultured with or without $10^{-4}$ M carnosine for up to 10 days. On days 5, 7 and 10, the medium was changed to fresh medium containing carnosine with or without 1.0 μg/ml noggin (G-T, Minneapolis, MN, USA), and then incubation was continued for 24 h. As a control, the cells were incubated without carnosine and noggin for 24 h. After incubation, total RNA was isolated from each cultured cell with a kit, and semiquantitative RT-PCR was carried out using gene-specific primers for RUNX2/Cbfa1 or Sox9 as described previously.

### Statistical analysis

All the experiments were performed in triplicate. Each value represents the mean ± SD. The significance of differences was determined using Bonferroni's modification of Student's *t*-test. Differences with $p$-values $< 0.05$ were considered significant.

## Results

### Effect of carnosine on cell proliferation

A logarithmic growth phase occurred in all cultures between days 1 and 3 after seeding and human periodontal ligament cells reached confluence on day 7. Cell proliferation was not affected by the addition of carnosine for up to 10 days in culture (Fig. 1).

### Effect of carnosine on ALPase activity

In the controls, ALPase activity increased gradually by day 7 of culture, and decreased on day 10. In the presence of $10^{-4}$ or $10^{-5}$ M carnosine, ALPase activity increased gradually with the culture days for up to 10 days, and enhanced significantly on days 7 and 10 of culture (Fig. 2).

### Effect of carnosine on expression of BMP receptors

The expression of BMP receptors was determined on day 7 of culture (Fig. 3). Of the BMP type I receptors, the human periodontal ligament cells expressed BMPR-IB and ActR-I, whereas no BMPR-IA expression was

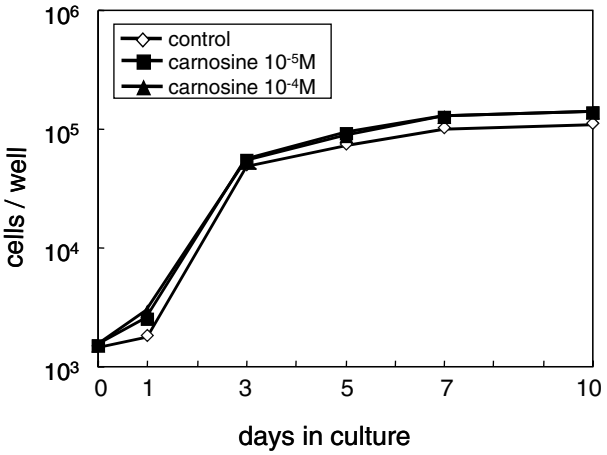


*Fig. 1.* Effect of carnosine on cell proliferation. Human periodontal ligament cells were cultured with or without $10^{-4}$ or $10^{-5}$ M carnosine for up to 10 days, and cell numbers were determined with a cell counting kit on days 1, 3, 5, 7 and 10 of culture.

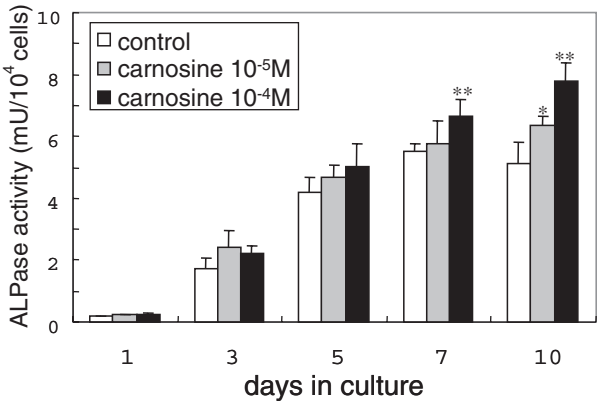


*Fig. 2.* Effect of carnosine on alkaline phosphatase (ALPase) activity. Human periodontal ligament cells were cultured with or without $10^{-4}$ or $10^{-5}$ M carnosine for up to 10 days, and ALPase activity was determined on days 1, 3, 5, 7 and 10 of culture. The data shown are the mean ± SD for four separate experiments; $*p < 0.05$, $**p < 0.01$, carnosine treatment vs. control.

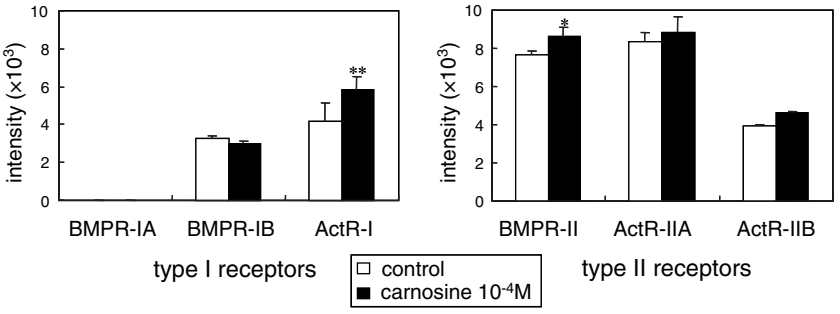


*Fig. 3.* Effect of carnosine on the mRNA expressions of type I and type II bone morphogenetic protein (BMP) receptors. Human periodontal ligament cells were cultured with or without $10^{-4}$ M carnosine, and expressions were determined by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) on day 7 of culture. The PCR products were visualized with ethidium bromide and quantified by the intensity of staining. The data shown are the mean ± SD for four separate experiments; $*p < 0.05$, $**p < 0.01$, carnosine treatment vs. control.

detected, irrespective of the addition of carnosine. The expression of ActR-I in cell culture was increased significantly by $10^{-4}$ M carnosine. By contrast, the expression of BMPR-IB was not affected by the addition of carnosine.

Of the BMP type II receptors, the human periodontal ligament cells expressed BMPR-II, ActR-IIA and ActR-IIB. The expression of BMPR-II in cell culture was increased significantly by $10^{-4}$ M carnosine. By contrast, the expression of ActR-IIA and ActR-IIB was not affected by the addition of carnosine.

### Effect of carnosine on phosphorylation of Smad1

The phosphorylation of Smad1 was determined by western blot analysis on day 7 of culture (Fig. 4). An immunoreactive band that corresponded to phosphorylated Smad1 was recognized at a molecular mass of approximately 65 kDa, and was increased by the addition of $10^{-4}$ and $10^{-5}$ M carnosine.

### Effect of noggin on expression of RUNX2/Cbfa1 and Sox9

The expression of RUNX2/Cbfa1 and Sox9, incubated in the medium containing carnosine with or without noggin or without carnosine and noggin, was determined on days 5, 7 and 10 of culture (Fig. 5). Noggin reduced significantly carnosine-induced up-regulation of RUNX2/Cbfa1 and Sox9 to control levels on days 7 and 10 of culture and day 7 of culture, respectively.

## Discussion

Recently, a crucial transcription factor for osteoblast or chondroblast differentiation was identified (20). RUNX2/Cbfa1, an osteoblast-specific transcription factor, has been cloned and implicated as a major regulator of osteoblast differentiation and gene expression (21). RUNX2/Cbfa1 belongs to a family of three transcription factors containing a common DNA-binding runt domain (22). By contrast, Sox9, an important transcription factor that mediates differentiation to chondroblasts, has been cloned and

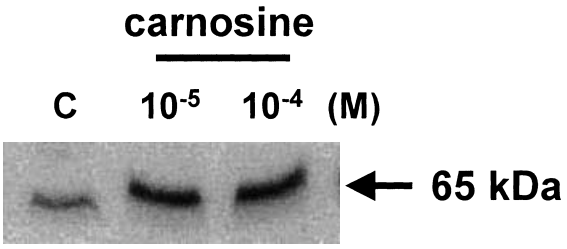


*Fig. 4.* Effect of carnosine on the phosphorylation of Smad1. Human periodontal ligament cells were cultured with or without $10^{-4}$ or $10^{-5}$ M carnosine, and immunoreactive proteins were determined using western blot analysis on day 7 of culture.

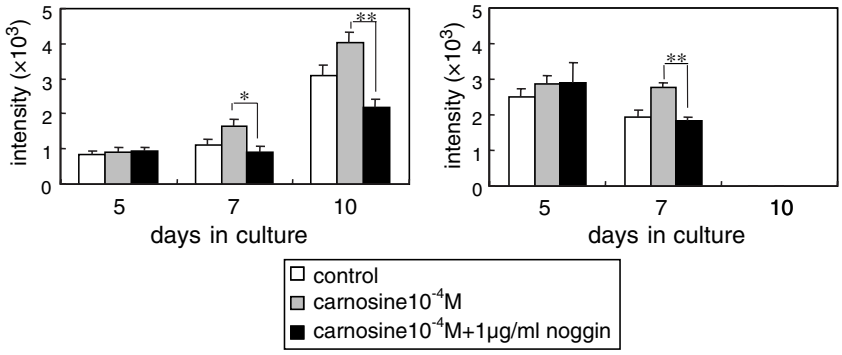


*Fig. 5.* Effect of noggin on carnosine-induced up-regulation of runt-related transcription factor-2/core binding factor α-1 (RUNX2/Cbfa1) (left) and Sox9 (right) mRNA. Human periodontal ligament cells were cultured with or without $10^{-4}$ M carnosine for up to 10 days. On days 5, 7 and 10, the medium was changed to fresh medium containing carnosine with or without 1.0 μg/ml noggin, or without carnosine and noggin as a control. After incubation for 24 h, the expression of RUNX2/Cbfa1 or Sox9 was determined by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), and the PCR products were visualized with ethidium bromide and quantified by the intensity of staining. The data shown are the mean ± SD for four separate experiments; $*p < 0.05$, $**p < 0.01$, noggin treatment vs. carnosine-induced expression.

implicated as a life cycle of chondrocytes in the developing skeleton (23).

Maeno *et al.* (12) demonstrated that gene expression of RUNX2/Cbfa1 in human periodontal ligament cells increased with prolonged culture with carnosine, and was notably higher than control values on day 10 of culture. By contrast, gene expression of Sox9 was only recognized on days 5 and 7 of culture; the expression increased upon addition of carnosine, and no expression was detected on day 10, irrespective of the presence of carnosine. These results suggest that carnosine may promote mesenchymal differentiation of human periodontal ligament cells to osteoblasts or chondroblasts *in vitro*.

BMP-2, BMP-4 and BMP-7, members of the transforming growth factor β (TGF-β) superfamily, are the main growth factors that promote the differentiation of mesenchymal cells into osteoblasts or chondroblasts (24, 25). Ducy *et al.* (23) demonstrated that BMP-7 induced the expression of RUNX2/Cbfa1 mRNA before the induction of osteocalcin mRNA in C3H10T1/2 cells from early mouse embryos. BMP-2 also increased RUNX2/Cbfa1 mRNA expression in an immortalized human bone marrow stromal cell line (hMC2-6) (26), C2C12 cells (27), and 2T3 cells (28).

With regard to human periodontal ligament, Maeno *et al.* (12) demonstrated that BMP-7 expression in human periodontal ligament cells increased on days 1–5 of culture in the initial stage of cell culture with carnosine, and BMP-2 expression increased slightly on day 1 and day 10 of culture with carnosine. By contrast, BMP-4 expression was very weak, and no effect of carnosine was observed.

Nishimura *et al.* (29) reported that BMP-2 induced RUNX2/Cbfa1 mRNA in C2C12 myoblasts, and that this induction was abolished by the overexpression of dominant-negative Smad1, Smad4 and Smad5, which are signal-transducing molecules of the TGF-β superfamily. In addition, Hanai *et al.* (30) demonstrated that Smad1 or Smad5 and RUNX2/Cbfa1 formed complexes, indicating an intimate interaction between these molecules during osteoblast differentiation.

On the basis of above facts, we hypothesized that the increase in RUNX2/Cbfa1 or Sox9 expression with carnosine stimulation may mediate via BMP-2 or BMP-7 produced by the cells. Consequently, the effect of carnosine on the expression of BMP receptors was examined in order to verify that the expression of RUNX2/Cbfa1 or Sox9 is increased by autocrine action via BMP-2 or BMP-7. Human periodontal ligament cells expressed BMPR-IB, ActR-I, BMPR-II, ActR-IIA, and ActR-IIB. The expression of ActR-I and BMPR-II receptors was significantly increased in cell cultures with carnosine, and the phosphorylation of Smad1, a signal-transducing molecule of BMP-2, BMP-4 and BMP-7 (30), was increased in cell cultures with carnosine. Furthermore, Noggin, a factor known to inhibit BMP-2, BMP-4 and BMP-7 functions (31–33), reduced carnosine-induced up-regulation of RUNX2/Cbfa1 or Sox9 mRNA, suggesting that BMP2 and BMP-7 were responsible for up-regulating RUNX2/Cbfa1 gene expression in human periodontal ligament cells.

In conclusion, carnosine may enhance RUNX2/Cbfa1 and Sox9 expression of human periodontal ligament cells via the autocrine action of BMP-2 or BMP-7 produced by the cells.

## Acknowledgements

This study was supported by funding from a Grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to promote multidisciplinary research projects, and

Grants-in-Aid of Scientific Research from the Japan Society for the Promotion of Science (No. 13307059 and No. 13671985). This study was also supported by the Uemura Fund and the Sato Fund, of Nihon University School of Dentistry (2003), and by special research grants from the Promotion and Mutual Aid Corporation for Private Schools, Japan (2003).

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