Biosci. Biotechnol. Biochem., **71** (11), 2680–2687, 2007

Promotion by Collagen Tripeptide of Type I Collagen Gene Expression in Human Osteoblastic Cells and Fracture Healing of Rat Femur

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Received May 11, 2007; Accepted August 2, 2007; Online Publication, November 7, 2007
[doi:10.1271/bbb.70287]

Peptides produced by the enzymatic degradation of collagens are reported to have various activities of biological and medical interest. The mechanisms underlying their actions are, however, poorly understood. We have produced, by collagenase digestion of type I collagen, a highly purified, non-antigenic, and low allergenic tripeptide fraction (collagen tripeptide, Ctp). We report here the effects of Ctp on the *in vivo* bone fracture healing and *in vitro* calcification of osteoblastic cells. An oral administration of Ctp to rats with a femur fracture accelerated the fracture healing. Ctp apparently stimulated the calcification of human osteoblastic cells in culture. This osteotrophic effect was accompanied by a significant increase in type I collagen protein production and its mRNA levels. DNA microarray and quantitative RT-PCR analyses demonstrated that Ctp upregulated the bone-specific transcription factor, Osterix, suggesting that the induction of type I collagen gene expression by Ctp was mediated by upregulation of this factor.

Key words: collagen peptide; Osterix; osteoblast; fracture healing; DNA microarray

Collagen is a major component of the extracellular matrix in almost all tissues of vertebrates and is produced in large quantities as a byproduct of the livestock industry. Collagen molecules form triple helical structures from three α chains which comprise a highly repetitive sequence of Gly-Xaa-Yaa (Xaa and Yaa are arbitrary but are often occupied by proline, hydroxyprolin, and alanine). This unique sequence and structure enables collagen molecules to show resistance to general proteinases. The degradation of collagen and preparation of collagen peptides thus require collagenolytic proteinases, collagenases.

Collagen peptides (the enzymatic degradation products of collagens) have recently been shown to have several biological activities, and have been used as preservatives[1)] and immunotherapeutic agents.[2,3)] Although these applications of collagen peptides have become very popular, the mechanisms underlying their actions are not fully understood.

Using a bacterial collagenase that degrades the peptide bonds of collagen at the amino-terminal end of Gly,[4)] we have prepared from porcine type I collagen a highly purified, non-antigenic, and low-allergenic tripeptide fraction (collagen tripeptide, Ctp) containing Gly-Xaa-Yaa sequences.[5)] We recently found that rats that were orally administered with Ctp showed accelerated recovery of fractured bones.[6)]

We show in this paper the osteotrophic effects of Ctp on bone fracture healing in a rat model, and examine the effects of Ctp on type I collagen synthesis and on overall gene expression in human osteoblastic cells in culture.

Materials and Methods

Materials. Ctp was prepared as previously described.[4)] The purity of Ctp is expressed as the content of tripeptides in the fractions, this being estimated from the peak integral of the absorbance at 215 nm by HPLC with a Superdex Peptide gel filtration column (Pharmacia Biotech, Sweden). Briefly, collagen peptide was prepared from porcine skin collagen by collagenase digestion. The digest was deionized with an ion exchanger (DAION type SK, Mitsubishi Chemical, Tokyo, Japan) and then passed through a 0.2-μm filter, the

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Abbreviations: Ctp, collagen tripeptide; *Col1a1*, type I collagen α-chain gene; Osx, Sp7 transcription factor/Osterix; Runx2/Cbfa1, runt-related transcription factor 2; Msx2, Msh homeobox 2; Dlx5, distal-less homeobox 5; BMP2, bone morphogenetic protein 2; Pept1, peptide transporter 1; TOR, target of rapamycin

resulting fraction (20% purity) being used for *in vivo* experiments. Further purification was performed for *in vitro* experiments by eliminating the endotoxins, using an ACP-0013 module (Asahi Chemical, Tokyo, Japan) and fractionation of the tripeptide fraction by reverse-phase HPLC. The tripeptide content was more than 90% after this purification. A D-MEM/F-12 medium (1:1) was purchased from Sigma (St. Louis, MO, USA), and HEPES, G418 and L-ascorbic acid were from Nacalai Tesque (Kyoto, Japan). CELLYARD Beads™ comprising high-purity hydroxyapatite were from Pentax (Tokyo, Japan), rat normal feed, CRF-1, was from Oriental Yeast (Tokyo, Japan), and oligonucleotides were produced by Texas Genomics Japan (Tokyo, Japan).

Oral administration of Ctp to the fracture-model rats. The procedures for the animal experiments were approved by the institutional animal care and use committee guidelines of the Central Research Institute of Jellice Co., Ltd. We used 7-week-old IGS male rats as the bone-fracture model. The middle of the left femur of each rat was cut in an operation, and the fractured bone was fixed with wires. These rats were divided into three groups ($N = 10$ in each group). From day 1 after the operation, the rats of each group received 0, 80, or 500 mg/kg/day of Ctp (20% purity) for 12 weeks (84 days). The left (fractured) and right (control) femurs were then extirpated, and the progression of fracture healing was observed by soft X-ray exposure. The bone strength (stiffness and breaking load) was measured by the three-point bending test[7] with MZ-500D apparatus (Maruto, Tokyo, Japan). An isolated bone was horizontally fixed at both ends in the machine, and a probe was moved down (20 mm/min) at the mid-point of the bone. The stiffness and breaking load parameters were recorded when the bone was broken according to the manufacturer's instructions.

Cell culture. Human osteoblastic hFOB1.19 cells were purchased from American Tissue Culture Collection (Manassas, VA, USA). The hFOB1.19 cells were immortalized by transfection with the temperature-sensitive SV40 large T antigen gene.[8] Cell proliferation is permitted at 34 °C and restricted at 39 °C. At the restrictive temperature, the cells show osteoblastic phenotypes including the expression of osteoblast-specific genes and increased collagen production. The cells were maintained at 34 °C in a D-MEM/F-12 medium containing 10% (v/v) FBS, 15 mM HEPES, and 0.3 mg/ml of G418 (growth medium). For the mineralization assay, the cells were seeded at 50,000 cells/cm^2 in 6-well plates and were cultured at 34 °C in the growth medium. When the cells became confluent, the culture medium was replaced with a D-MEM/F-12 medium containing 1% (v/v) FBS, 15 mM HEPES, 0.3 mg/ml of G418, and 50 μg/ml of L-ascorbic acid (assay medium), and the cells were incubated with Ctp (highly purified, >90%) at 10 μg/ml at 37 °C for 7 days. The culture medium was replaced with a fresh assay medium containing 10 μg/ml of Ctp every 2 days. After 7 days of culture, the cells were fixed with ice-cold 70% (v/v) ethanol and stained for calcium deposition by the Alizarin Red S method as described previously.[9] For the collagen production, cells were seeded at 50,000 cells/cm^2 in 6-well plates and were cultured at 34 °C in the growth medium. When cells became confluent, the culture medium was replaced with the assay medium, and the cells were incubated at 39 °C for 24 h. Ctp was then added to the culture medium at 10 μg/ml, and the cells were further incubated for 48 h at 39 °C.

Production of type I collagen. To quantify collagen production by the hFOB1.19 cells, we detected the c-terminal extension peptides (CICP) released into the culture medium. Collagen is synthesized as procollagen containing extension peptides at the N- and C-termini. After the procollagen molecule is secreted from cells, these extension peptides are cleaved and released as soluble peptides. Thus, these peptides provide a stoichiometric representation of collagen synthesis.[10] The culture medium of the hFOB1.19 cells was centrifuged at 16,000 × *g* and assayed with a Metra™ CICP EIA kit (Quidel, San Diego, CA, USA). Total protein concentration was determined by the Bradford method[11] with bovine serum albumin as a standard.

Quantitative real-time RT-PCR analysis. Cells for the gene expression assay were seeded at 50,000 cells/cm^2 in 6-well plates and were cultured at 34 °C in the growth medium. When the cells became confluent, the culture medium was replaced with the assay medium and the cells were incubated at 39 °C for 24 h. The cells were then incubated with Ctp (highly purified, >90%) at the indicated concentrations for the indicated periods at 39 °C. Total RNA was isolated from the treated and untreated hFOB1.19 cells with an RNeasy mini-kit (Qiagen, Chatsworth, CA, USA). For the type I collagen expression in the presence of hydroxyapatite, the cells were incubated with hydroxyapatite beads (1 mg/ml) at 39 °C for 24 h, and then Ctp was added to the culture medium at various concentrations, before the cells were further incubated at 39 °C for 24 h. The gene expression of type I collagen and other genes was analyzed by quantitative real-time PCR, using an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). Primers were designed by Primer Express software (Applied Biosystems) as shown in Table 1. Total RNA of 10–100 ng and 0.1–1.0 μM sequence-specific primers were used for the reaction in 20 μl with a one-step SYBR® RT-PCR kit (Takara, Kyoto, Japan). The reaction mixtures were incubated at 42 °C for 15 min for reverse transcription and then at 95 °C for 2 min for denaturation; the thermal cycling parameters were 95 °C/5 s for denaturation, and 60 °C/30 s for annealing and elongation (40 cycles). After detection by an ABI

Table 1. Primer Sequences for Quantitative Real-Time RT-PCR

Gene	Sense primer	Antisense primer
Col1a1	AGGGCCAAGACGAAGACATCCC	TGTCGCAGACGCAGATCCG
Osx	GCAAGAGGTTCACTCGTTCGGATG	TGTTTGCTCAGGTGGTCGCTTC
Runx2/Cbfa1	TGGACGAGGCAAGAGTTTCACC	CTTCTGTCTGTGCCTTCTGGGTTC
Msx2	CCGCCTCGGTCAAGTCGGAAA	AGGGCTCATATGTCTTGGCGG
Dlx5	TGCCGACTATAGCTACGCTAGCTCC	CACTTCTTTCTCTGGCTGGTTGGTG
Bmp2	AACACTGTGCGCAGCTTCC	CCTAAAGCTTGCATCTGTTCTC
Gapdh	ATCACCATCTTCCAGGAGCGAGA	TGGTGAAGACGCCAGTGGACTC

Prism 7700 sequence detector, the data were analyzed by SDS 2.1 software (Applied Biosystems), and the average Ct value was calculated from triplicate reactions. The average Ct value was normalized by that of the internal standard gene, GAPDH, and the relative mRNA expression levels of samples with added Ctp and the control were calculated.

DNA microarray analysis. A DNA microarray analysis was performed by using GeneChip® Human Genome U133 Plus 2.0 and the analysis system (Affymetrix, Santa Clara, CA, USA). Total RNA was extracted from Ctp-treated (10 μg/ml, 24 h) or untreated hFOB1.19 cells with an RNeasy mini-kit, and the concentration and purity were checked by a UV spectrophotometric analysis. Using a One-cycle Target Labeling kit (Affymetrix), total RNA was reverse-transcribed with a T7-Oligo (dT) primer and SuperScript II reverse transcriptase, this being followed by second-strand cDNA synthesis with DNA polymerase, and the cDNAs were used for *in vitro* transcription with T7 RNA polymerase. The resulting complimentary RNAs were biotinylated, fragmented, and hybridized to GeneChip® arrays according to the manufacturer's instructions. After washing and staining the probe arrays with a Fluidics station 450 (Affymetrix), they were scanned by a GeneChip® 3000 scanner (Affymetrix). Gene expression data were normalized to a target intensity of 500, and were then used for a comparative analysis between the control and Ctp-treated samples.

Results and Discussion

Effects of Ctp on rat bone fracture healing

To assess the effects of Ctp on bone formation *in vivo*, we used an artificial rat bone fracture model.[7] During fracture healing, the broken areas generally are initially filled with calluses, this being followed by mineralization from the periosteum, reduction, and finally, disappearance of the calluses.[7,12]

The rats with a fracture of the left femur were divided into three groups of 10 each that respectively received a daily oral administration of 0, 80, or 500 mg/kg/day of Ctp for 12 weeks (84 days). Each femur was then extirpated and exposed to soft X-rays. Figure 1A presents typical pictures of the femurs showing no conjugation or a clear fracture line and large callus (a)

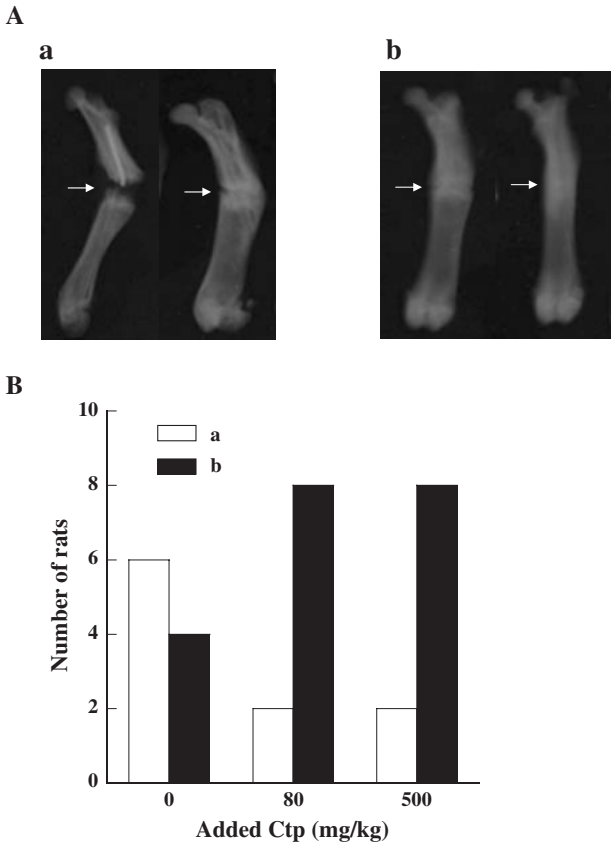

Fig. 1. Effects of Ctp on Fracture Healing of the Rat Femur.

Rats whose left femur had been cut in an operation received daily oral Ctp in the indicated amounts for 12 weeks (84 days) and the femurs were then extirpated and observed by soft X-rays.[6] A, (a), typical pictures of femurs showing no conjugation or a clear fracture line and a large callus; (b), typical pictures of femurs with an obscure or undetectable fracture line and cortex continuity. Arrows indicate the fracture position. B, Fracture healing of rats with and without Ctp. Open and closed bars indicate the numbers of rats with an unhealed femur (a) and those with a healed femur (b), respectively.

and of the femurs with an obscure or undetectable fracture line and cortex continuity (b). As shown in Fig. 1B, six of the ten rats that received no Ctp showed incomplete fracture healing; two of these showed no conjugation of the fractured bone and large calluses on both sides (Fig. 1Aa, left) and four showed conjugated bone but still with large calluses and clear fracture lines (Fig. 1Aa, right), indicating that they were still at an early stage of fracture healing. In contrast, the rats that

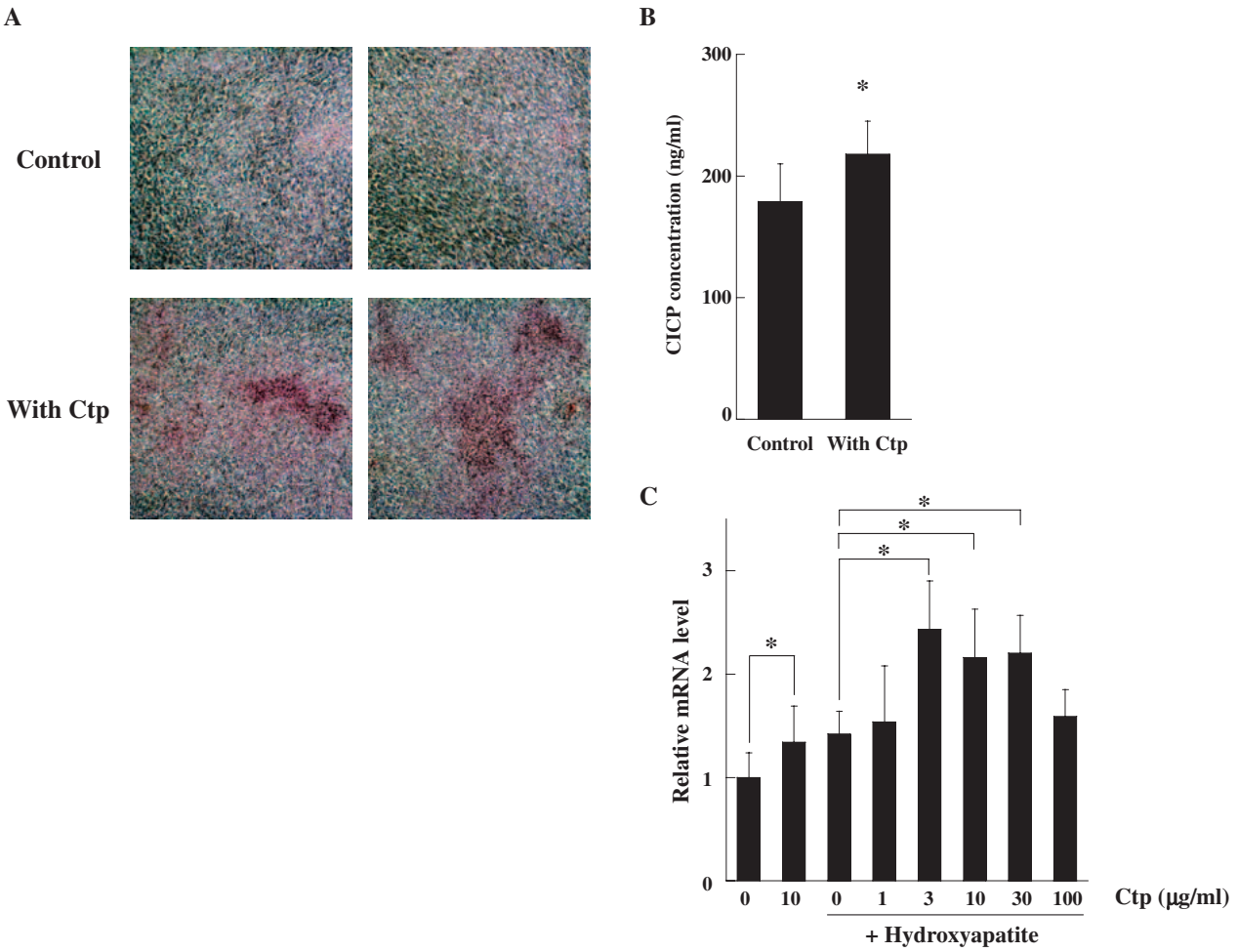

Fig. 2. Effects of Ctp on Human Osteoblastic Cells.

A, Mineralization of hFOB 1.19 cells. Cells were cultured at 37 °C for 7 days with and without 10 μg/ml of Ctp. Mineralization was analyzed by staining with Alizarin Red S. Typical pictures are shown; the red-stained areas correspond to the mineralized matrix. B, Type I collagen protein production. hFOB1.19 cells were cultured under a restrictive condition for 48 h with and without 10 μg/ml of Ctp. The C-terminal extension peptide (CICP) concentration in each culture supernatant was analyzed by ELISA. Columns and bars indicate the mean (N = 3) and standard deviation, respectively, $^{*}p < 0.05$. C, Type I collagen mRNA level. hFOB1.19 cells were treated with the indicated concentrations of Ctp under restrictive conditions for 24 h in the presence or absence of 1 mg/ml of hydroxyapatite beads. *Col1a1* mRNA expression was analyzed by quantitative real-time RT-PCR. The intensity of each *Col1a1* mRNA signal was normalized with that of GAPDH and related to that of the control. Columns and bars indicate the mean (N = 3) and standard deviation, respectively, $^{*}p < 0.05$.

received 80 or 500 mg/kg/day of Ctp showed significantly accelerated fracture healing (Fig. 1B). Only two of the ten rats showed large calluses and clear fracture lines; the others had an obscure or undetectable fracture line (Fig. 1Ab). Compared with the control, the healed bone of the Ctp-treated rats showed a significant improvement in stiffness (0.86 ± 0.07 [500 mg/kg/day of Ctp] *vs.* 0.47 ± 0.10 [untreated]) and a breaking load (0.86 ± 0.11 [500 mg/kg/day of Ctp] *vs.* 0.62 ± 0.11 [untreated]). Each value is expressed as the ratio of the left femur (fractured and healed) to the right femur (control), and the normal value is 1.0.[6)]

Effects of Ctp on the mineralization of a human osteoblastic cell culture

To examine the effects of Ctp on osteoblastic cells, we first looked at the mineralization of hFOB cells in the presence of Ctp. After 7 days of culture, we detected an apparent increase of mineralized nodules in the cultures treated with 10 μg/ml of Ctp (Fig. 2A). The results suggest that Ctp accelerated bone fracture healing though the stimulation of osteoblast calcification. We looked at the proliferation and alkaline phosphatase activity of the Ctp-treated cells, but they were essentially unchanged when compared with the untreated cells (data not shown). These results indicate that Ctp affected factors other than these in the stimulation of mineralization.

Type I collagen expression in the Ctp-treated human osteoblastic cells

We next examined collagen production in the Ctp-treated hFOB1.19 cells. Type I collagen is a major component of osteoblastic cells and is involved in the

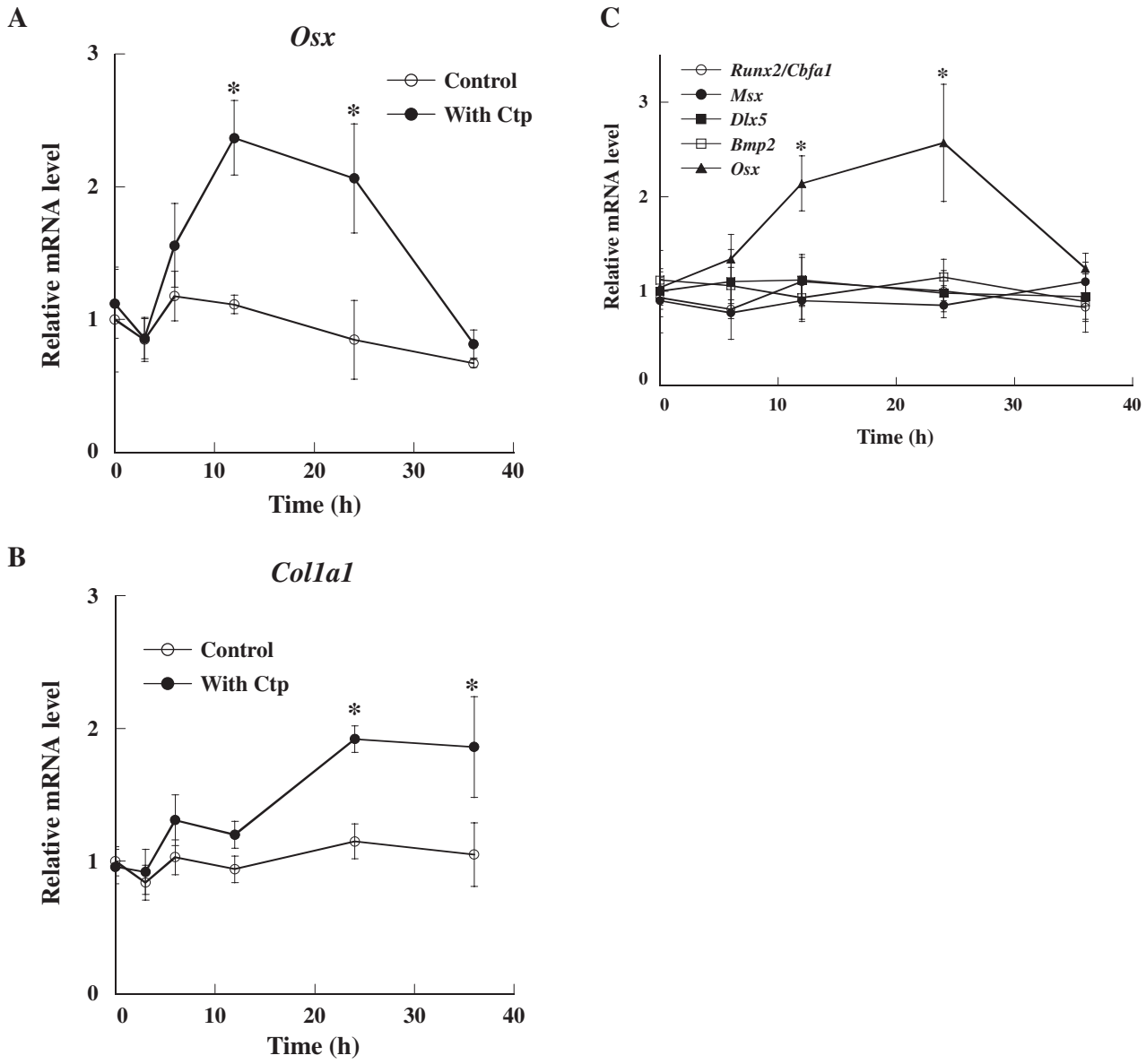

Fig. 3. Time-Course Characteristics of the mRNA Levels of *Osx* (A), *Col1a1* (B) and *Osx*-Related Genes (C).

A and B, hFOB1.19 cells were treated with (filled circles) and without (unfilled circles) 10 μg/ml of Ctp for the indicated periods, and total RNA was isolated and analyzed by quantitative real-time RT-PCR. Each value is the mean (N = 3) and bars indicate the standard deviation, $^{*}p < 0.05$ (*vs.* the value without Ctp). C, mRNA expression analysis of *Osx*-related genes. hFOB1.19 cells were treated with 10 μg/ml of Ctp for the indicated periods, and total RNA was isolated and analyzed by quantitative real-time RT-PCR. Runx2/Cbfa1, runt-related transcription factor 2; Msx2, Msh homeobox 2; Dlx5, distal-less homeobox 5: Bmp2, bone morphogenetic protein 2; Osx, Sp7 transcription factor/Osterix. Each value is the mean (N = 3) and bars indicate the standard deviation, $^{*}p < 0.05$ (*vs*. the value at 0 h).

mineralization process.[13] hFOB1.19 cells were cultured in the presence or absence of 10 μg/ml of Ctp for 2 days, and type I collagen production was analyzed by measuring CICP in the culture medium. As shown in Fig. 2B, Ctp significantly increased the release of CICP (1.3-fold), indicating that Ctp induced a 1.3-fold increase of type I collagen protein production. We then examined the type I collagen mRNA levels in Ctp-treated cells by quantitative real-time RT-PCR. The levels of mRNA for the α-chain of type I collagen were significantly higher (1.3–1.7-fold) in the presence of 10 μg/ml of Ctp (Figs. 2C and 3B), suggesting that Ctp stimulated type I collagen protein production through the upregulation of type I collagen α-chain gene (*Col1a1*) expression.

We further examined the effects of Ctp on *Col1a1* expression in the presence of high-purity hydroxyapatite beads in hFOB1.19. Hydroxyapatite [$Ca_{10}(PO_4)_6OH_2$] has been reported to increase *Col1a1* expression in mouse osteoblastic cells.[14] It is thought that the attachment of osteoblasts to hydroxyapatite may induce osteoblastic cell differentiation at an early stage and stimulate the synthesis of extracellular matrix proteins, including type I collagen.[14,15] As expected, Ctp gave 1.7-fold higher *Col1a1* mRNA levels at 3 μg/ml (1.7-

Table 2. Affymetrix Analysis of Genes Upregulated by Ctp in hFOB1.19 Cells

Gene name	Gene bank	No.-fold change
Sp7 transcription factor/Osterix	NM_152860	3.92
Far upstream element (FUSE) binding protein 1	NM_003902	3.13
Runt-related transcription factor 1, translocated to 1	NM_004349	2.84
Ets variant gene 6	NM_001987	2.69
Calreticulin	NM_004343	2.69
Cullin-associated and neddylation-dissociated 1	NM_018448	2.55
Checkpoint suppressor 1	NM_005197	2.48
Endothelial PAS domain protein 1	NM_001430	2.41
Peroxisome proliferative activated receptor γ	NM_005037	2.38
AT rich interactive domain 1B (SWI1-like)	NM_017519	2.32
PRKC, apoptosis, WT1, regulator	NM_002583	2.31
Enhancer of zeste homolog 2	NM_004456	2.28
Zinc finger protein 544	NM_014480	2.24
SWI/SNF related, matrix associated, actin dependent regulator of chromatin	NM_003070	2.20
Nuclear antigen Sp100	NM_003113	2.19
Runt-related transcription factor 1	NM_001754	2.18
Signal transducer and activator of transcription 3	NM_003150	2.18
Nuclear receptor subfamily 4, group A, member 2	NM_006186	2.15
Forkhead box P1	NM_032682	2.14

Each value is the mean of three independent experiments.

fold) in the presence of hydroxyapatite when compared with the control (Fig. 2C).

These results suggest that the stimulation of osteoblast mineralization by Ctp can be accounted for by the induction of type I collagen production by Ctp.

Affymetrix microarray analysis for the genes upregulated by Ctp

To examine the effects of Ctp on overall gene expression, we performed a DNA microarray analysis of the gene expression in Ctp-treated hFOB1.19 cells by using an Affymetrix GeneChip® array and detected 169 probe sets that were upregulated more than 2.12-fold. Among them, Gene Ontology annotations identified 19 genes that are thought to be involved in transcriptional regulation. These genes were reproducibly upregulated by the Ctp treatment in three independent experiments, with Sp7 transcription factor/Osterix (Osx) showing the greatest change in expression (3.92-fold on average) (Table 2).

Osx has been reported to be an essential factor for osteoblast differentiation.[16] It contains a C_2H_2-zinc finger motif, binds to the *Col1a1* promoter and regulates the expression of major bone matrix protein genes, including *Col1a1*, bone sialoprotein, and osteocalcin.[16] Osx knockout mice die soon after birth and show impaired bone formation and reduced expression of *Col1a1*.[16] Along with these findings, our microarray results suggest that Osx is involved in the Ctp upregulation of *Col1a1* gene expression. A quantitative real-time RT-PCR analysis clearly demonstrated that the induction of *Col1a* gene expression by Ctp was preceded by the upregulation of Osx expression (Fig. 3A and B).

Expression of Osx-related genes

Osx null mice show a phenotype similar to that of runt-related transcription factor 2 (Runx2/Cbfa1) knockout mice.[17] In Runx2/cbfa1 null embryos, Osx is not expressed, and osteoblast differentiation is arrested, whereas Runx2/Cbfa1 is expressed in Osx null cells. These observations suggest that Osx acts downstream of Runx2/Cbfa1.[16] To examine whether the Runx2/Cbfa1 signaling pathway would be affected by Ctp, we analyzed the mRNA levels of Runx2/Cbfa1, Msh homeobox 2 (Msx2), distal-less homeobox 5 (Dlx5),[18] and bone morphogenetic protein 2 (BMP2).[19] BMP2 initiates osteoblastic cell differentiation and acts upstream of Runx2/Cbfa1, while Msx2 and Dlx5 are known to act downstream of Runx2/Cbfa1.[16,19–21] As shown in Fig. 3C, the mRNA levels of these genes remained unchanged, and only Osx was upregulated in the Ctp-treated hFOB1.19 cells. These results suggest that Ctp did not directly affect the expression of the Runx2/Cbfa1 or BMP2-induced signaling pathways.

The mechanisms underlying the osteotrophic effects of Ctp are currently unknown. How Ctp activates *Osx* gene expression is the most important issue to be clarified. Two possible mechanisms can be considered. First, Ctp stimulates *Osx* gene expression through a peptide hormone-like mechanism, *i.e.*, through binding to a cell surface receptor. Second, Ctp enters the osteoblast cells and directly or indirectly activates *Osx* gene expression through intracellular peptide sensor proteins similar to the target of rapamycin (TOR). TOR has been reported to sense amino acids and short peptides, and to stimulate the transcription, translation, and degradation of the proteins involved in protein synthesis.[22] We do not have any data suggesting which possibility is more likely, but it should be noted here that we detected the expression of mRNA for peptide transporter 1 (Pept1) in hFOB1.19 cells with the DNA

microarray analysis of this study (data not shown). Pept1 has been reported to be specifically expressed in intestinal epithelial cells and to mediate the uptake of short peptides such as di- and tripeptides.[23] We have previously prepared ^{3}H-labeled Gly-Pro-Hyp (Gly-Pro-Hyp is a representative tripeptide in Ctp and Pro was labeled with ^{3}H), and observed that when rats orally received this radiolabeled tripeptide, radioactivity could be detected in the blood and in those tissues that actively produce collagens, such as bone, skin, tendon, and kidney, more rapidly than the rats which received [^{3}H]Pro.[6] We, however, have not yet confirmed whether the radiolabeled tripeptide remained intact in the blood and tissues, this being an urgent issue to be examined for understanding the *in vivo* effect of Ctp. Collagen-derived di- and tripeptides have recently been identified in the blood of human volunteers who had orally received a gelatin hydrolysate (average molecular weight of 5,000 to 14,500) and the tripeptides had Gly at their carboxyl termini (Xaa-Hyp-Gly).[24,25] Their sequences were distinct from those of our Ctp (Gly-Xaa-Yaa) and it is unclear whether the Xaa-Hyp-Gly tripeptides had osteotrophic effects or other biological activities.

In conclusion, this study has revealed the osteotrophic effects of Ctp. Ctp can accelerate bone fracture healing in a rat model and stimulate type I collagen production by osteoblastic cells in culture through upregulation of the transcription factor, Osx. Although more studies are needed to clarify the mechanisms of Ctp action, the present findings show new regulatory features in osteoblast differentiation and bone formation that may assist in developing preventive measures against bone loss and treatments for bone fractures.

Acknowledgment

We thank Dr. Yasuhito Ishigaki for his kind guidance with the Affymetrix microarray analysis. We also thank Kazumi Tanaka for her assistance. This work was supported by grants for promoted research from Kanazawa Medical University (nos. S2004-15 and S2005-2) and by from the Ministry of Education, Culture, Sports, Science and Technology of Japan grant-aid for young scientists (B) (no. 16790858).

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