Calcif Tissue Int (2000) 67:391–399
DOI: 10.1007/s002230001150

Calcified Tissue International

The Effect of Carboxyl-Terminal Propeptide of Type I Collagen (c-Propeptide) on Collagen Synthesis of Preosteoblasts and Osteoblasts

M. Mizuno, R. Fujisawa, Y. Kuboki

Department of Biochemistry, School of Dentistry, Hokkaido University, Sapporo, Japan 060

Received: 6 August 1999 / Accepted: 9 May 2000 / Online publication: 22 September 2000

Abstract. Recently we found that the carboxyl-terminal propeptide of type I collagen (c-propeptide) is a major secretory protein of osteoblasts. Mature osteoblasts secreted 64 nM c-propeptide, and it was reported that 40 nM c-propeptide inhibited collagen synthesis at 80% of the control level. In this study, we investigated the effect of c-propeptide on collagen synthesis of preosteoblasts and osteoblasts, and found that preosteoblasts downregulated collagen synthesis by 40 nM c-propeptide, but osteoblasts were not affected by the same condition. When the binding activities of c-propeptide for preosteoblasts and osteoblasts were compared, osteoblasts showed weak affinity to c-propeptide compared with preosteoblasts, and the number of receptors for c-propeptide decreased in osteoblasts. These results imply that a decrease of receptors in osteoblasts might reduce the sensitivity of osteoblasts to c-propeptide.

Key words: C-propeptide — Osteoblasts — Preosteoblasts — Collagen synthesis

The carboxyl-terminal propeptide of type I collagen (c-propeptide) is a major protein secreted by osteoblasts, and is present in bone [1]. All collagens are initially produced as a precursor, procollagen [2], and procollagen contains the amino- and carboxyl-peptides in the molecule [3]. During the conversion of procollagen to collagen, the propeptides are cleaved by specific proteases [4]. These peptides are considered to be readily degradated after they are released from procollagen. However, the aminoterminal propeptide, and c-propeptide of type I collagen are present in bone [1, 5, 6].

C-propeptide is necessary for the formation of the triple helix structure of collagen [7]. Furthermore, it has been postulated that c-propeptide suppresses collagen synthesis in a feedback manner, and 40 nM c-propeptide causes 80% reduction of collagen synthesis, whereas the synthesis of noncollagenous proteins is not affected in IMR-90 fibroblasts [8]. We found that osteoblastic MC3T3-E1 cells produced 64 nM c-propeptide, and that collagen synthesis was not inhibited, though this concentration is higher than that causing 80% suppression of collagen synthesis [8]. In this study, we investigated the mechanism of the lower sensitivity of osteoblasts to c-propeptide and found that osteoblasts had a decreased number of receptors for c-propeptide, which may reduce the sensitivity of osteoblasts to c-propeptide.

Correspondence to: M. Mizuno

Materials and Methods

Preparation of Carboxyl-Terminal Propeptide of Type I Collagen (c-propeptide) from Conditioned Medium of Osteoblastic MC3T3-E1 Cells

C-propeptide was purified from conditioned media of osteoblastic MC3T3-E1 cells, which formed mineralized tissues, as described previously [1]. Briefly, conditioned media was subjected to Concanavalin A chromatography equilibrated with 0.5 M NaCl/20 mM Tris (pH 7.4), and the bound fraction was eluted from the column by the above buffer containing 0.2 M α-methyl mannoside. The final purification was carried out by CM-cellulose chromatography and Sephacryl S-200 gel filtration, and the purity of c-propeptide was verified by SDS-polyacrylamide gel electrophoresis. After the sample containing c-propeptide was dialyzed against phosphate-buffered saline, it was stored at -20°C until use.

Cell Culture

The osteoblastic MC3T3-E1 cells were cultured in α minimum essential medium (αMEM) containing 10% fetal calf serum [9–11]. 1×10^5 cells were plated in a 35-mm dish and cultured in a humidified atmosphere of 5% CO_2 (v/v) in air at 37°C. They reached the confluent stage on day 3 after the inoculation, and formed mineralized tissues at days 15 to 20. To elucidate the effect of c-propeptide on collagen synthesis of osteoblasts, c-propeptide (20 or 40 nM) or 50 μl of whole anti-c-propeptide serum was added to the medium and cultured for 24 hours.

Measurement of Alkaline Phosphatase (ALP) Activity and DNA Contents

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 10 mM Tris buffer (pH 7.6), and the homogenates were used for ALP activity assay. One unit was estimated to be 1.4 mg of p-nitrophenol liberated from p-nitrophenyl phosphate in 15 minutes [12]. DNA content was determined using bisbenzimidazole (Hoechst 33258) [13]. Briefly, cell pellets were homogenized with 50 mM Na_2HPO_4/2.0 M NaCl (pH 7.4) and mixed with Hoechst 33258. The intensity of fluorescence was measured at an excitation wavelength of 358 nm and emission wavelength of 458 nm. All standard stock solution was stored at -20°C until use.

Measurement of Newly Synthesized Proteins by Incorporation of ^{3}H-Proline

The osteoblastic MC3T3-E1 cells were cultured with medium containing 10 μ Ci of ^{3}H-proline and 10% fetal calf serum (FCS) for 4 hours. The medium and cell-matrix were harvested, and the cell-matrix was homogenized in phosphate-buffered saline (PBS) using a glass homogenizer. The medium and homogenate were combined and precipitated by the addition of 10% trichloroacetic acid and 1% tannic acid. Precipitates were harvested by centrifugation at 12,000 rpm for 10 minutes at 4°C, and were washed twice with 1% trichloroacetic acid and 0.1% tannic acid. The radioactivity of precipitates was measured with a liquid scintillation counter.

Measurement of Collagen Synthesis in Cell-Matrix

The content of newly synthesized collagen in the cell-matrix was shown as the radioactivity of collagenase-digestible protein (CDP), which was measured by the bacterial collagenase digestion assay [14]. Briefly, osteoblastic MC3T3-E1 cells were cultured in medium containing 10 μ Ci of ^{3}H-proline and 10% FCS for the last 4 hours of a 24-hour culture, and cell-matrix layers were digested by highly purified bacterial collagenase (Advance Biofactures Corporation, Form III). Then 10% trichloroacetic acid and 1% tannic acid were added to the reaction mixture, and it was maintained at 4°C overnight. After centrifugation at 12,000 rpm for 10 minutes at 4°C, the radioactivity of the supernatant was determined using a liquid scintillation counter.

Measurement of c-Propeptide Content in Medium by Antibody Capture ELISA Assay

The content of c-propeptide in the conditioned medium was determined by the antibody capture assay [15]. Briefly, a microtiter plate was coated with c-propeptide (5 pmol/well) and blocked by 3% bovine serum albumin (BSA). The conditioned medium was incubated with an anti-c-propeptide antibody for an appropriate time, then added to a precoated microtiter plate. Bound antibodies were detected using protein A-conjugated ALP with p-nitrophenyl phosphate as the substrate.

Cell-Attachment Analysis

For this analysis, 100 μl of c-propeptide solution diluted to 1, 2, 5, 10, 20, 40, 60 pmol/well with PBS was applied to a 96-well microtiter plate. The plate was maintained at 4°C overnight to fix c-propeptide onto the plate. Sites on the plastic for nonspecific binding were blocked with 200 μl of 3% BSA maintained for 30 minutes at room temperature. The osteoblastic MC3T3-E1 cells were detached with 0.012% (w/v) pronase, and 0.02% (w/v) EDTA, and then washed once with serum-free αMEM. Cells were resuspended to 1×10^5 cells/ml, and 100 μl of cell suspension was applied to each well. Cells were cultured for 1 hour in a humidified atmosphere of 5% (v/v) CO_2 in air at 37°C, and then wells were washed with PBS three times to remove nonadherent cells. Cell number was measured by colorimetric assay using Alamer Blue [16].

Binding Assay

C-propeptide was labeled by the lactoperoxidase method with ^{125}I [17], and free iodine was removed by ultrafiltration using a Centricon (Amicon Co., Japan). Cells (2.5×10^5) were seeded on a 48-multiwell plate, and cultured until they attached completely. Then cells were washed with PBS three times and incubated for 20 minutes with PBS preheated at 37°C and containing 5 mg/ml of BSA. After cells were washed with ice-cold PBS, binding assays were performed in the presence of 0.2 ml/well of ice-cold serum-free medium containing an appropriate concentration of ^{125}I-labeled c-propeptide and 0.1% BSA. Cells were maintained at 4°C for 1 hour under shaking, and incubation was terminated by gentle aspiration of the medium and washing cells four times with ice-cold PBS. Bound radioactivity was measured in a gamma counter after solubilization of cells by incubation for 10 minutes with 0.5 ml of 1% Triton X-100 in PBS, followed by 0.5 ml of PBS alone. Nonspecific binding was measured in the presence of ^{125}I-labeled c-propeptide and a 100-fold excess amount of native c-propeptide. All binding assays were carried out in duplicate and experiments were repeated twice.

Electron Microscopic Observation

Cells were fixed using 1% fresh formalin and 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) at room temperature. After postfixing in 1% osmium tetraoxide in the same buffer for 2 hours, they were dehydrated and embedded in Quetol-812 (Nissin EM, Japan). Sections 80 nm thick were cut using a PORTER BLUM MT-2 ultramicrotome, mounted on copper grids and stained with uranyl acetate and lead citrate. The observation was carried out using a Hitachi H-800 transmission electron microscope operated at 75 kv.

Results

Mineralized Tissue Formation and Collagen Synthesis by Osteoblastic MC3T3-E1 Cells

The osteoblastic MC3T3-E1 cells in the subconfluent stage showed a fibroblastic form (Fig. 1A), and the cellular outline changed to a mosaic appearance after cells reached confluence (Fig. 1B). Cells continuously grew and formed multiple cell layers, and cell outline became obscure at day 5 after confluence (Fig. 1C). Mineralized tissues were formed during the period from 15 to 20 days after confluence (Fig. 1D). Electron microscopic observation revealed matrix vesicles containing needle-like crystals, and crystals precipitated on collagen fibers (Fig. 1E). These crystals were identified as hydroxyapatite by their electron diffraction pattern (data not shown). ALP activity, which is an osteoblastic phenotype, was not detected at the subconfluent stage; however, its activity increased time dependently after the confluent stage (Fig. 2). At day 20 after confluence, cells formed mineralized tissues, and ALP activity was fourfold that of cells at day 5. Cells were cultured with ^{3}H-proline to measure *de novo* synthesis of proteins. ^{3}H-proline incorporation increased time dependently, and at day 20, cells showed twofold higher incorporation than cells at the subconfluent stage (Table 1). The ratio of collagen in newly synthesized proteins of cell matrix was estimated by a specific collagenase-digestion assay. At days 5 and 20, the ratios of collagen in synthesized proteins of cell-matrix were 9 and 33%, respectively; however, collagen synthesis was not detected at day 2 (Fig. 3).

These results indicated that cells at day 20, after the confluent stage, formed mineralized tissues and expressed osteoblastic phenotypes; furthermore, collagen synthesis was active, whereas at day 5, ALP activity and collagen synthesis were weak, which meant that the cells remained in the preosteoblast stage.

Appearance of Carboxyl-Terminal Propeptide of Type I Collagen (c-propeptide) in the Differentiated Osteoblasts, and Purification of c-Propeptide from Conditioned Media

We cultured osteoblastic MC3T3-E1 cells for 20 days, and

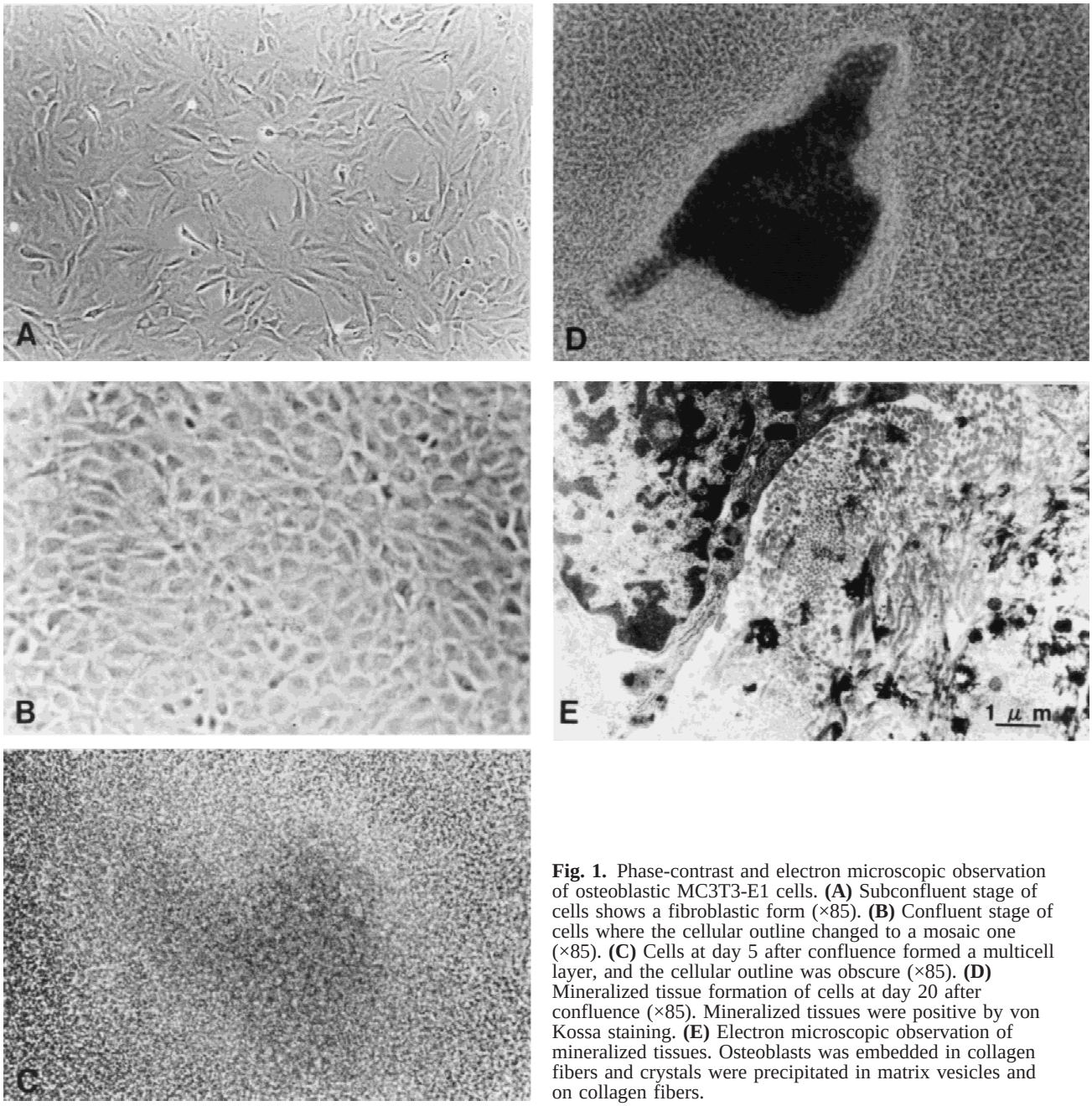

Fig. 1. Phase-contrast and electron microscopic observation of osteoblastic MC3T3-E1 cells. **(A)** Subconfluent stage of cells shows a fibroblastic form (×85). **(B)** Confluent stage of cells where the cellular outline changed to a mosaic one (×85). **(C)** Cells at day 5 after confluence formed a multicell layer, and the cellular outline was obscure (×85). **(D)** Mineralized tissue formation of cells at day 20 after confluence (×85). Mineralized tissues were positive by von Kossa staining. **(E)** Electron microscopic observation of mineralized tissues. Osteoblasts was embedded in collagen fibers and crystals were precipitated in matrix vesicles and on collagen fibers.

medium was changed to ^{3}H-proline (10 μ Ci/ml) containing serum-free medium, and we analyzed radiolabeled proteins present in medium and cell-matrix fraction by a fluorographic method. In medium, we found procollagen (α1 and 2), and the c-propeptide α1 chain (35 kDa) and α2 chain (33 kDa) (Fig. 4), as described previously [1]. However, amino-terminal propeptide (15~20 kDa) was not present. In cell-matrix fraction, collagen (α1 and 2) was present as a major synthesized protein (Fig. 4). We purified c-propeptide from the condition medium by a previously described procedure [1], and verified its purity by SDS-polyacrylamide gel electrophoresis (Fig. 5). Next we raised the polyclonal antibody by the injection of purified c-propeptide into rabbits. We investigated the specificity of the antibody by western blotting, and found that the antibody reacted with c-propeptide; however, the antibody did not recognize procollagen in the medium (Fig. 6). This result may be due to the conformational change of c-propeptide after releasing from procollagen.

Next we measured c-propeptide content in the medium by an antibody capture ELISA assay. At the subconfluent stage and at day 2 after confluence, c-propeptide was not detected, but at days 5 and 20, the contents of c-propeptide were 12 nM and 64 nM, respectively (Fig. 7). C-propeptide is considered to inhibit collagen synthesis [8, 18], and 80% of collagen synthesis of fibroblasts was inhibited by 40 nM

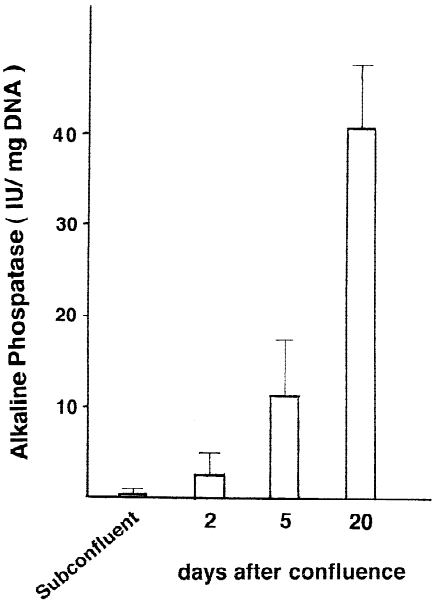

Fig. 2. Alkaline phosphatase (ALP) activity of osteoblastic MC3T3-E1 cells. ALP activity increased time dependently after confluence. Data shown were ± SD and were evaluated from five dishes.

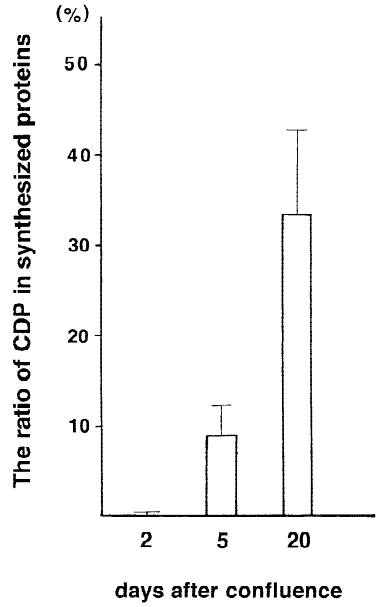

Fig. 3. The ratio of collagen in newly synthesized proteins of cell matrix was evaluated from the content of collagenase-digestible protein (CDP). Data shown are mean ± SD, and were evaluated from three experiments.

Table 1. ^{3}H-proline incorporation into newly synthesized proteins

MC3T3-E1 cells	dpm/dish
Subconfluent stage	213,643 ± 2,427
2 days after confluence	226,593 ± 1,978
5 days after confluence	331,428 ± 3,095
20 days after confluence	419,131 ± 3,723

Data shown were mean ± SD, and were evaluated from three dishes

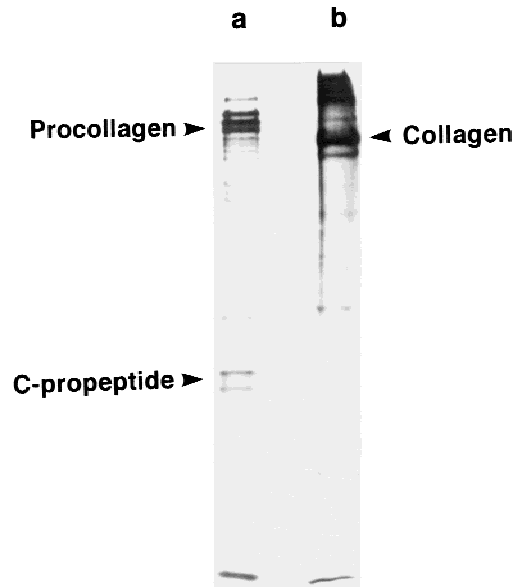

Fig. 4. Fluorography of the conditioned medium **(a)** and cell-matrix faction **(b),** which was separated by SDS-polyacrylamide gel electrophoresis using 10% of polyacrylamide gel under reducing conditions. In the medium, procollagen and c-propeptide were detected, and collagen was recognized in the cell-matrix fraction.

c-propeptide [8]. However, our results indicated that osteoblasts produced collagen actively even though they produced 64 nM c-propeptide. Therefore, we speculated that osteoblasts had resistance to the inhibitory effect of c-propeptide on collagen synthesis.

Inhibition of Collagen Synthesis by c-Propeptide

The osteoblastic MC3T3-E1 cells cultured for 5 days and 20 days after confluence (designated preosteoblasts or osteoblasts, respectively, in this article) were treated by 20 or 40 nM c-propeptide for 24 hours, and the collagen synthesis was examined. In preosteoblasts, collagen synthesis was downregulated dose dependently by c-propeptide, and 40 nM c-propeptide suppressed collagen synthesis at 60% of the control level; however, osteoblasts were not affected by 40 nM c-propeptide (Fig. 8). Osteoblastic MC3T3-E1 cells grew slowly after the confluent stage, and formed multicell layers. Therefore, it was presumed that the discrepancy of inhibitory effect on cells was due to the difference of cell numbers. In fact, the number of osteoblasts was approximately twice that of preosteoblasts. We harvested preosteoblasts and osteoblasts from culture dishes by pronase and EDTA treatment and adjusted the cell number to that of preosteoblasts.

After cells attached on dishes, the effect of c-propeptide was examined. Preosteoblasts showed downregulation of collagen synthesis by 40 nM c-propeptide at 67% of the control level; however, osteoblasts were not affected (Table 2). These results indicated that osteoblasts were less sensitive to c-propeptide than preosteoblasts. Downregulation of

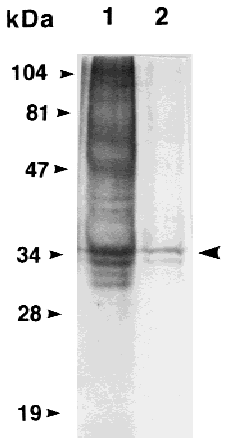

Fig. 5. Purification of c-propeptide from the conditioned medium. C-propeptide was separated with the procedure described in Materials and Methods. Lane 1; concanavalin A bound fraction separated from the conditioned medium. Lane 2; purified c-propeptide (indicated by arrowhead) which contained neither procollagen nor collagen. SDS-polyacrylamide gel electrophoresis using 12.5% of polyacrylamide gel was carried out under reducing conditions.

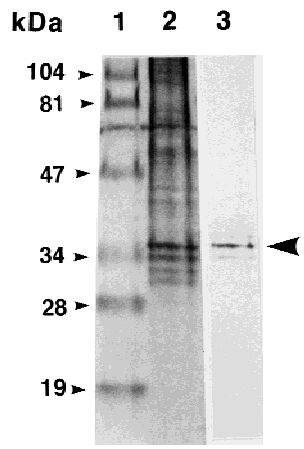

Fig. 6. Western blotting of serum-free conditioned medium with anti-c-propeptide. Lane 1; prestained standard proteins. Lane 2; Coomassie Brilliant Blue staining of serum-free medium. Lane 3; immunostaining with anti-c-propeptide. The antibody we raised, stained c-propeptide; however, the procollagen in the medium did not react with the antibody.

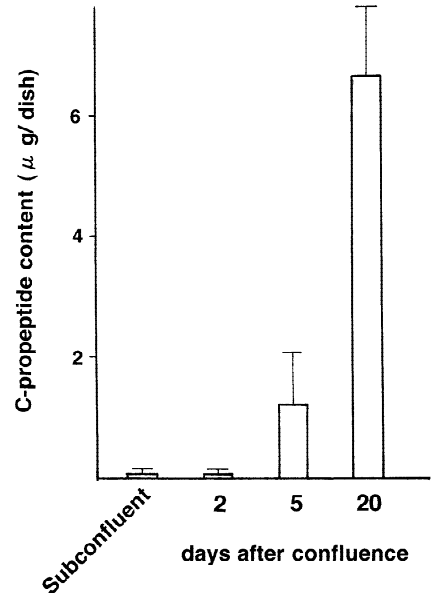

Fig. 7. C-propeptide content in 1 ml of conditioned medium, which was measured by antibody capture ELISA assay. At day 20 after confluence, 64 nM (6.6 μg/ ml) of c-propeptide was detected in the medium. Data shown are mean ± SD, and were evaluated from five samples.

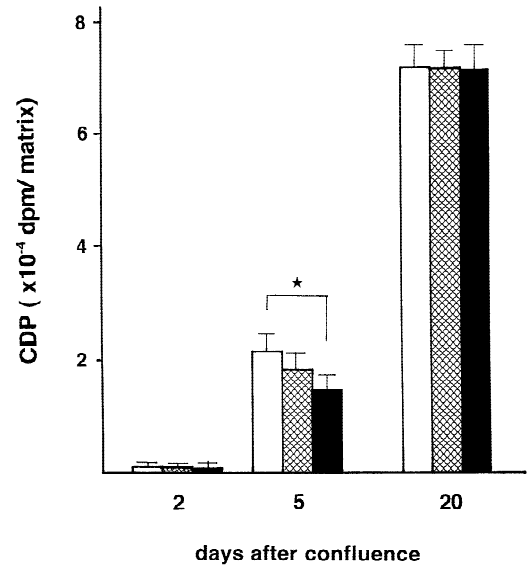

Fig. 8. Collagenase-digestible protein (CDP) content in the cell matrix. Cells at day 2, 5, and 20 after confluence were cultured with vehicle (empty bar), 20 nM (cross-hatched bar), and 40 nM (filled bar) c-propeptide for 24 hours. Collagen synthesis of cells at day 5 was inhibited by c-propeptide dose dependently. Star; $P < 0.05$. Data shown are mean ± SD, and were evaluated from three dishes.

collagen synthesis could occur as a result of damage to cells. To confirm that the effect of c-propeptide was not due to injury to cells, the effect of an anti-c-propeptide antibody was examined. Cells were cultured for 5 days after confluence, and medium was changed to one containing 40 nM of c-propeptide and 50 μl of whole anti-c-propeptide serum. The antibody abolished the inhibitory effect of c-propeptide, and collagen synthesis did not change (Table 3).

Binding of c-Propeptide to Receptors of Cell Membrane

It is reported that the inhibition of collagen synthesis by c-propeptide occurs by direct regulation of collagen gene transcription after the internalization and association of nuclear components [19]. For the internalization, c-propeptide should bind the cell membrane, and α2β1 integrin has been identified as a main receptor of c-propeptide [20]. To study the binding of c-propeptide to cells, we investigated the cell attachment to c-propeptide-coated dishes, because integrin receptors mediate cell attachment to extra-

Table 2. Effect of c-propeptide on collagen synthesis of preosteoblasts and osteoblasts

	Collagenase-digestible protein (CDP)		
	Control (dpm/matrix)		C-propeptide (40 nM) (dpm/matrix)
Preosteoblasts	19,564 ± 2,736	*	13,072 ± 2,648
Osteoblasts	39,564 ± 1,866	N.D.	38,092 ± 2,338

N.D. no difference
Data shown were mean ± SD, and were evaluated from three dishes
* $P < 0.01$

Table 3. Effect of anti-c-propeptide on collagen synthesis suppressed by c-propeptide[a]

	CDP content (dpm/matrix)
Control	16,749 ± 1,736
C-propeptide	11,772 ± 2,648
C-propeptide+ anti-c-propeptide	16,434 ± 2,048
Anti-c-propeptide	16,589 ± 2,712

Data shown were mean ± SD, and were evaluated from three dishes
[a] The antibody abolished the action of c-propeptide

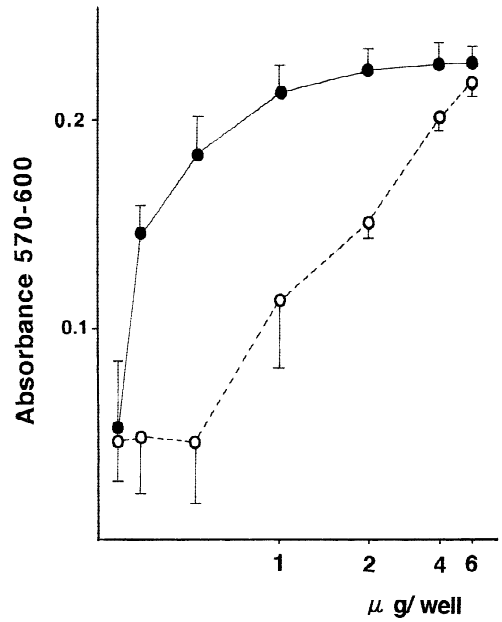

Fig. 9. Adhesive activity of preosteoblasts (filled cricle) and osteoblasts (open circle) to c-propeptide. Preosteoblasts showed high affinity to c-propeptide compared with osteoblasts. Data shown are mean ± SD, and were evaluated from five samples.

cellular matrix. Various amounts of c-propeptide (1, 2, 5, 10, 20, 40, 60 pmol/dish) were placed on dishes, followed by blocking with 3% BSA, and preosteoblasts or osteoblasts were inoculated onto the dishes. Both cells showed binding activity to c-propeptide dose dependently; however, osteoblasts showed lower affinity than preosteoblasts (Fig. 9). Next, we investigated binding of ^{125}I-labeled c-propeptide to osteoblasts and preosteoblasts. After cells attached to the dishes, the medium was changed so as to contain various amounts of ^{125}I-labeled c-propeptide and 0.1% BSA, and then incubated at 4°C for 1 hour. Radioactivity bound to cells increased dose dependently, and there were fewer number of receptors of osteoblasts (1.9×10^5 binding site/cell) than of preosteoblasts (2.4×10^5 binding site/cell) (Fig. 10). However, affinity of receptors to c-propeptide showed no definite difference between osteoblasts (Kd = 151 nM) and preosteoblasts (Kd = 141 nM) (Fig. 10). The binding of ^{125}I-labeled c-propeptide to cells was abolished by excess amounts of cold c-propeptide (data not shown). These results indicated that osteoblasts had low affinity to c-propeptide, and that this might be due to the decrease in number of receptors.

Discussion

Osteoblasts and Collagen Synthesis

Collagen synthesis of osteoblastic MC3T3-E1 cells increased as the osteoblastic differentiation proceeded. When cells formed mineralized tissues, collagen accounted for 33% of newly synthesized proteins of cell matrix. It is reported that 95% of collagen newly synthesized by osteoblastic MC3T3-E1 cells is type I collagen [21]. Type I collagen is the major extracellular matrix protein, accounting for approximately 85% of the protein in bone [22]. Type I collagen enhances the expression of osteoblastic phenotypes [23], and rat UMR201 preosteoblastic cells develop a more mature phenotype when cells are cultured on type I collagen gels [24]. The collagen matrix is believed to inhibit the proliferation and to promote the osteblastic phenotype [25]. When collagen synthesis is inhibited by eliminating ascorbic acid or adding a collagen-synthesis inhibitor, osteoblastic differentiation is markedly suppressed [26–28]. These findings indicate that collagen synthesis and collagen-osteoblast interaction are crucial for osteoblastic differentiation.

Production of c-Propeptide by Osteoblasts and the Specificity of Antibody Against c-Propeptide

All collagens are initially produced as the precursor, procollagen [2], and this is converted to collagen by removing amino- and c-propeptides by specific proteases [3, 4]. We investigated the protein synthesis by the incorporation of ^{3}H-proline into proteins, and found procollagen and c-propeptide in the medium and collagen in the cell-matrix fraction (Fig. 4). However, a definite amount of procollagen was not recognized when SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the serum-free conditioned medium was carried out (Fig. 6). This discrepancy between the fluorographic analysis and SDS-PAGE pattern of serum-free medium is due to the difference of proline content in the molecule. The helical domain of collagen contains an approximately fivefold higher content of proline than that of c-propeptide [29]. These results indicate that the content of procollagen in the conditioned medium is less compared with that of c-propeptide. Furthermore, the antibody against c-propeptide did not react with procollagen in the medium

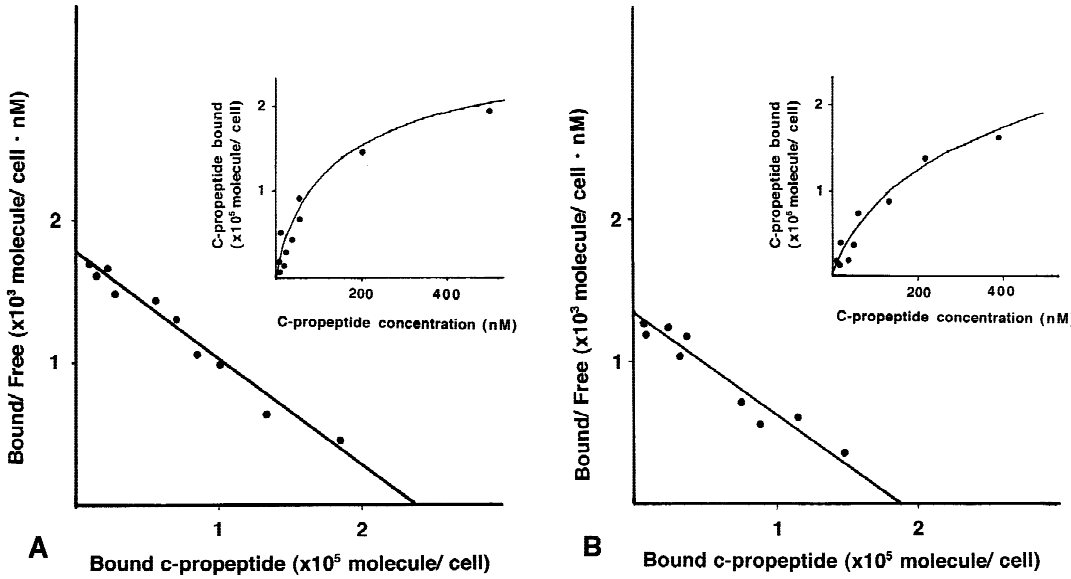

Fig. 10. Schachard plot analysis of ^{125}I-labeled c-propeptide binding to MC3T3-E1 osteoblastic cells. 2.5×10^5 cells of preosteoblasts **(A)** and osteoblasts **(B)** were cultured for 1 hour at 4°C in the presence of various concentrations of ^{125}I-labeled c-propeptide. Inner panel shows the specific binding of ^{125}I-labeled c-propeptide to cells. Each data point represents the mean of duplicated determination, and the experiments were repeated twice.

(Fig. 6). This finding may be due to the difference of three-dimensional conformation of c-propeptide present in procollagen and released form. Our results imply that the antibody is useful for the measurement of c-propeptide content in the conditioned medium.

In our experiment, amino-terminal propeptide was not observed, and similar results have been obtained using human fetal osteoblasts [30]. It was speculated that processing of amino-terminal propeptide from procollagen occurs slowly, though the precise mechanisms are unknown. Amino-terminal propeptide and carboxyl-terminal propeptide are present in bone [5, 6]. These findings indicate that both propeptides remain in bone.

Function of c-Propeptide for Osteoblasts

In this study, we demonstrated that osteoblastic MC3T3-E1 cells produced 64 nM c-propeptide. This concentration is higher than that causing 80% suppression of collagen synthesis in IMR-90 fibroblasts [8]. Thus, we speculated that the sensitivity of osteoblasts against c-propeptide was low. To confirm this hypothesis, we investigated the effect of c-propeptide on preosteoblasts and osteoblasts. Collagen synthesis of preosteoblasts was suppressed by 40 nM c-propeptide; however, osteoblasts were not affected by this condition. C-propeptide interacts with the cell membrane and can be integrated, then it migrates to the nucleus and associates with the nuclear components [19]. The binding with nuclear components might be the cause of feedback inhibition in a pretranscriptional inhibition manner [19]. On the other hand, Aycock et al. [18] reported that a synthetic peptide of a highly conserved portion of the c-propeptide of human pro-α2(1) procollagen inhibited collagen and fibronectin synthesis at the posttranscriptional level. Though the precise mechanism of feedback inhibition is obscure, the interaction of c-propeptide with the cell membrane is a prerequisite for the inhibitory effect. Recently, $\alpha 2\beta 1$ integrin was identified as a receptor of c-propeptide [20]. Davies et al. [31] found that c-propeptide also bound $\alpha 1$ $\beta 1$ integrin receptor, and that integrin -c-propeptide interactions were mediated by the α subunit of the A chain. However, cell attachment to c-propeptide was not affected when NHS fibroblasts were treated with an anti-$\alpha 1$ antibody, though cells expressed $\alpha 2\beta 1$ and $\alpha 1\beta 1$ integrin receptors [31]. These results indicated that the $\alpha 2\beta 1$ integrin receptor was a main receptor for c-propeptide. The osteoblastic MC3T3-E1 cells possess $\alpha 2\beta 1$ integrin receptor [32]. Therefore, c-propeptide might interact with $\alpha 2\beta 1$ integrin present on the cell membrane of MC3T3-E1 cells.

On the other hand, the $\alpha 2\beta 1$ integrin receptor is a receptor for collagen and recognizes the DGEA amino acid sequence of collagen [33]. The $\alpha 2\beta 1$ integrin recognition site on the $\alpha 1(1)$ collagen c-propeptide is composed of elements within residues 56-181 of the 249-residue propeptide, and this region does not contain a DGEA sequence [34]. Nor does this portion include residues 225-246, which inhibit collagen and fibronectin biosynthesis [18], or residues 212-216 (KTTKS), which stimulate the synthesis of extracellular matrix [35]. These findings imply that the integrin-binding sequence of c-propeptide differs from the binding sequence of collagen and regulating sequence for collagen biosynthesis.

To confirm that the lesser sensitivity of osteoblasts to c-propeptide was due to the lower osteoblast-c-propeptide interaction, we investigated the binding of c-propeptide to cells. C-propeptide supports cell attachment via integrin receptors [20]. We examined the attachment of preosteoblasts and osteoblasts to c-propeptide-coated dishes and found that preosteoblasts showed higher affinity to c-propeptide than osteoblasts. Furthermore, we measured the number of receptors and evaluated the affinity of receptors to c-propeptide in preosteoblasts and osteoblasts. Osteoblasts possessed fewer receptors than preosteoblasts, however, the Kd value was not different. These findings indicated that the lower sensitivity of osteoblasts to c-propeptide might be due

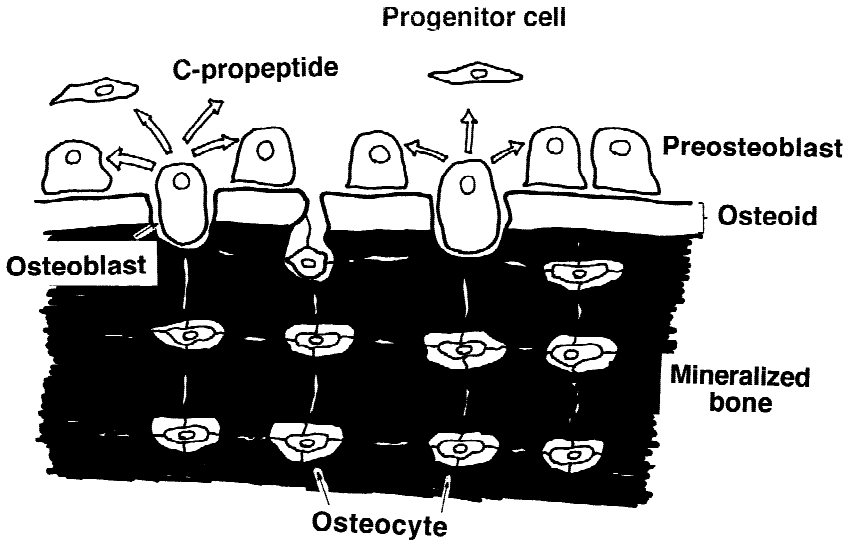

Fig. 11. Possible function of c-propeptide which is secreted from osteoblasts and binds preosteoblasts. Then collagen synthesis of preosteoblasts is suppressed, which retards osteoblastic differentiation.

to a decrease of receptors compared with preosteoblasts. Lynch et al. [23] reported that β 1 integrin gene expression was downregulated time dependently in a differentiation lineage. These results support our findings that osteoblasts expressed fewer receptors for c-propeptide. On the other hand, the competition of collagen and c-propeptide for the α 2 β 1 integrin receptor might also be involved. Collagen and c-propeptide are produced equimolarly during collagen synthesis. Collagen shows a 20-fold higher affinity to α 2 β 1 integrin receptors than c-propeptide [20]. Therefore, osteoblasts may bind collagen preferentially when collagen and c-propeptide are present simultaneously, and may reduce the sensitivity to c-propeptide.

Possible Function of c-Propeptide in Bone Formation

Bone is maintained by the balance of bone formation and bone resorption. In the periosteum, osteoprogenitor cells are present, and are induced to differentiate into osteoblasts by various stimulators (growth factors, cytokines, and hormones). Finally, they are embedded in the extracellular matrix, and differentiate into osteocytes. In the differentiation of osteoprogenitor cells into osteoblasts, all progenitor cells do not start to differentiate simultaneously. Some cells differentiate, and others remain in the progenitor stage. That is, ostoblastic differentiation of progenitor cells is not synchronized. We hypothesize that c-propeptide contributes to the dissynchronization of the differentiation. When osteoblasts produce extracellular matrix, c-propeptide is produced actively. C-propeptide migrates around osteoblasts, and may bind to preosteoblasts (Fig. 11). This binding will cause the suppression of collagen synthesis. Collagen synthesis and osteoblastic differentiation are tightly correlated because the inhibition of collagen synthesis suppresses osteoblastic differentiation [26–28]. Therefore, c-propeptide might retard the osteoblastic differentiation of preosteoblasts and may regulate the recruitment of osteoblasts.

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