Carboxyl-terminal propeptide of type I collagen (c-propeptide) modulates the action of TGF-β on MC3T3-E1 osteoblastic cells

Morimichi Mizuno*, Ryuichi Fujisawa, Yoshinori Kuboki

Department of Biochemistry, School of Dentistry, Hokkaido University, Kita 13 Nishi 7, Sapporo 060-8586, Japan

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Abstract Previously we found that the carboxyl-terminal propeptide of type I collagen (c-propeptide) is a major secretory protein of MC3T3-E1 osteoblastic cells. In this study, we found that c-propeptide suppresses collagen synthesis and alkaline phosphatase activity of MC3T3-E1 osteoblastic cells at the early-differentiated stage in a dose dependent manner. Mature osteoblasts did not respond to c-propeptide. These findings imply that c-propeptide modulates the function of osteoblasts at an early differentiation stage. Transforming growth factor-β (TGF-β) is stored in bone and released from bone matrix after the resorption by osteoclasts. We investigated the effect of c-propeptide on the action of TGF-β, and found that it enhanced the effect of TGF-β. We conclude that c-propeptide is a physiological modulator of TGF-β in bone metabolism.

Key words: c-Propeptide; Transforming growth factor-β; MC3T3-E1 osteoblastic cell

1. Introduction

Transforming growth factor-β (TGF-β) is a growth factor that modulates the growth and differentiation of various cell types [1,2]. It is produced by osteoblast [3], and stored in bone (200 μg/kg tissue weight) in a latent form [4]. TGF-β is released from bone during bone resorption [5,6], and stimulates bone formation in vivo [7,8]. However, in osteoblastic differentiation, TGF-β has been shown to prevent the expression of alkaline phosphatase (ALP) and osteocalcin, which are markers of mature osteoblasts [9,10]. These findings imply that the effect of TGF-β on osteoblasts is suppressed when osteoblastic cells differentiate into the mineralized tissue-forming stage [11].

Recently we observed that a carboxyl-terminal propeptide of type I collagen (c-propeptide) is a major secretory protein of osteoblasts, and that c-propeptide is stored in bone matrix [12]. All collagens are initially produced as precursors, called procollagen [13], and which contain extra amino- and carboxyl-peptides, which are called propeptide [14]. c-Propeptide is necessary for the formation of the triple helix structure of collagen [15]. During the conversion of procollagen to collagen, the propeptides are cleaved by a specific protease [16]. These peptides are considered to be readily broken after they are released from procollagen. However, the amino- and carboxyl-terminal propeptides of type I collagen are present in bone [12,17,18]. It has been reported that c-propeptide suppresses collagen synthesis [19,20], and promotes the attachment of osteoblasts [12]. However, its physiological function in bone metabolism is not known.

MC3T3-E1 osteoblastic cells display various osteoblastic phenotypes in a sequential manner under the long-term culture, and form mineralized tissue [21]. However, no mineralization was induced in the absence of ascorbic acid from the medium [22]. Moreover the expression of osteoblastic phenotypes is dependent on the production of type I collagen in osteoblastic cells [23].

In this study we investigated the effect of c-propeptide on the action of TGF-β on MC3T3-E1 osteoblastic cells, and found that c-propeptide enhanced the action of TGF-β.

2. Materials and methods

2.1. Biochemicals

^{125}I-labeled TGF-β and TGF-β were purchased from Amersham Pharmacia Biotech (Tokyo Japan) and Cosmo Bio. (Tokyo, Japan), respectively. Other reagents were purchased from Wako Pure Chemicals (Tokyo, Japan).

2.2. Preparation of c-propeptide from conditioned media of osteoblastic MC3T3-E1 cells

c-Propeptide was purified from the conditioned medium of osteoblastic MC3T3-E1 cells that formed mineralized tissue as described previously [12]. Briefly, the conditioned medium 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. The fraction containing c-propeptide was dialyzed against phosphate-buffered saline (PBS), and then it was stored at −20°C until use. Anti-c-propeptide polyclonal antibody was raised as described previously [12].

2.3. Cell culture

The osteoblastic MC3T3-E1 cells were cultured in α minimum essential medium (α MEM) which contains ascorbic acid (50 mg/l), with 10% fetal calf serum (FCS) [24]. Then 1×10^5 cells were plated in a 35 mm dish and cultured in a humidified atmosphere of 5% CO_2 (v/v) at 37°C. They reached the confluent stage on day 3 after the inoculation, and formed mineralized tissue at days 15–20. The effect of TGF-β was investigated in the presence of 10% FCS. TGF-β was dissolved in 17.5 mM acetic acid as a stock solution and was diluted with the medium to the concentration of 2 ng/ml. Cells were cultured in TGF-β-containing medium for 24 h, and then biochemical analysis was carried out.

2.4. Measurement of ALP activity and DNA contents

Cells were washed with PBS to remove serum proteins and were scraped from culture dishes. Cells were then homogenized in 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 min [25]. To determine DNA

*Corresponding author. Fax: (81)-11-706 4235.
E-mail: mmizuno@den.hokudai.ac.jp

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content, cell pellets were homogenized in 50 mM Na_2HPO_4/2.0 M NaCl (pH 7.4) and mixed with *bis*-benzimide (Hoechst 33258) [26]. The intensity of fluorescence was measured at an excitation wavelength of 356 nm and emission wavelength of 458 nm. All standard stock solutions were stored at −20°C until use.

2.5. Analysis of collagen synthesis

The newly synthesized collagen was revealed as the intensity of radioactivity of collagenase-digestible protein (CDP), which was measured by the bacterial collagenase digestion assay [27]. Briefly, osteoblastic MC3T3-E1 cells were cultured in medium containing 10 μCi of [^{3}H]proline and 10% FCS for the last 4 h of a 24 h 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 were maintained at 4°C overnight. After centrifugation at 12 000 rpm for 10 min at 4°C, the radioactivity of the supernatant was determined using a liquid scintillation counter.

2.6. TGF-β binding assay

For this assay, 2.5×10^5 cells 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 min with PBS preheated at 37°C and containing 5 mg/ml of bovine serum albumin. After the 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 TGF-β, and 0.1% bovine serum albumin. Cells were maintained at 4°C for 1 h 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 min with 0.5 ml of 1% Triton X-100 in PBS, followed by 0.5 ml of PBS alone. Non-specific binding was measured in the presence of ^{125}I-labeled TGF-β and a 100-fold excess amount of TGF-β. All binding assays were carried out in duplicate and experiments were repeated twice.

3. Results

3.1. The effects of c-propeptide on the collagen synthesis and ALP activity of MC3T3-E1 osteoblastic cells

We investigated the effects of c-propeptide on collagen synthesis and the ALP activity, which are the differentiation-associated phenotypes of osteoblast [28], of MC3T3-E1 osteoblastic cells. The cells formed mineralized nodules at days 15–20 after reaching confluence. MC3T3-E1 osteoblastic cells cultured for 5 days and 20 days after confluence (designated preosteoblasts or osteoblasts, respectively, in this article) were treated with 20 or 40 nM c-propeptide for 24 h, and the collagen synthesis and the ALP activity were examined. In preosteoblasts, collagen synthesis and ALP activity were downregulated by c-propeptide in a dose dependent manner (Table 1). The inhibition of collagen synthesis was consistent with previous findings [19,20]. However, neither parameter was affected by c-propeptide in osteoblasts (Table 1).

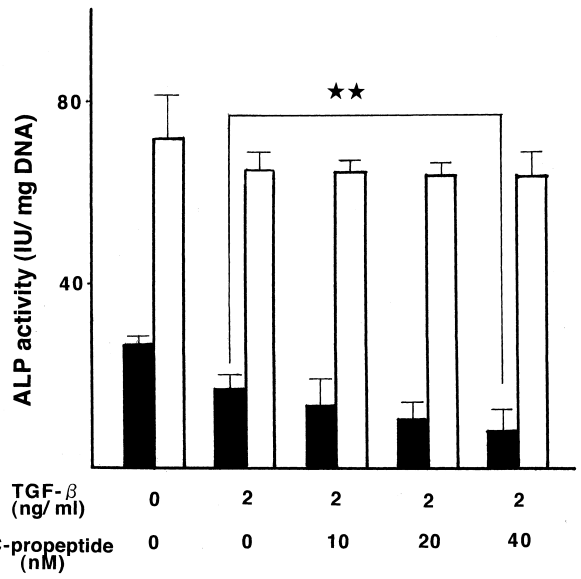

Fig. 1. The effect of c-propeptide on the action of TGF-β to ALP activity in preosteoblasts and osteoblasts. Preosteoblats that were cultured for 5 days after confluence (solid bars) and osteoblasts that were cultured for 20 days after confluence (open bars) were treated with TGF-β and c-propeptide for 24 h. Data were evaluated from four dishes and shown as mean ± S.D. $^{**}P<0.01$ by Student's *t*-test.

3.2. The effect of c-propeptide on the action of TGF-β on MC3T3-E1 osteoblastic cells

It has been reported that TGF-β suppresses ALP activity in MC3T3-E1 osteoblastic cell [29], and we found that c-propeptide inhibited ALP activity. Thus we investigated the effect of c-propeptide on the action of TGF-β. TGF-β suppressed the ALP activity of MC3T3-E1 osteoblastic cells; however, the magnitude of inhibition was weak in osteoblasts compared with preosteoblasts (Fig. 1). When c-propeptide was added to the medium containing TGF-β, the enzyme activity was downregulated in a dose dependent manner, and the enzyme activity was additively inhibited by TGF-β and c-propeptide in preosteoblasts (Fig. 1). In the presence of TGF-β (2 ng/ml) and c-propeptide (40 nM), ALP activity showed 50% inhibition (Fig. 1). On the other hand, c-propeptide did not affect the action of TGF-β in osteoblasts (Fig. 1). To confirm that the effect of c-propeptide was specific, cells were cultured with anti-c-propeptide antibody in medium containing TGF-β (2 ng/ml) and c-propeptide (40 nM). This treatment resulted in recovery of ALP activity (Table 2).

Table 1
The effect of c-propeptide on collagen synthesis and ALP activity of preosteoblast and osteoblast

	c-Propeptide (nM)	CDP ($\times10^4$ dpm/matrix)	ALP activity (IU/mg DNA)
Preosteoblast (5 days culture after confluence)	0	1.9 ± 0.32	28.7 ± 2.1
	20	1.7 ± 0.24	25.1 ± 2.9
	40	1.5 ± 0.21[a]	21.3 ± 2.6[a]
Osteoblast (20 days culture after confluence)	0	7.8 ± 0.25	70.0 ± 10.5
	20	7.7 ± 0.22	72.2 ± 11.3
	40	7.8 ± 0.23	71.5 ± 11.0

Values are evaluated from four dishes and shown as mean ± S.D.
[a] $P<0.05$ vs. vehicle by Student's *t*-test.

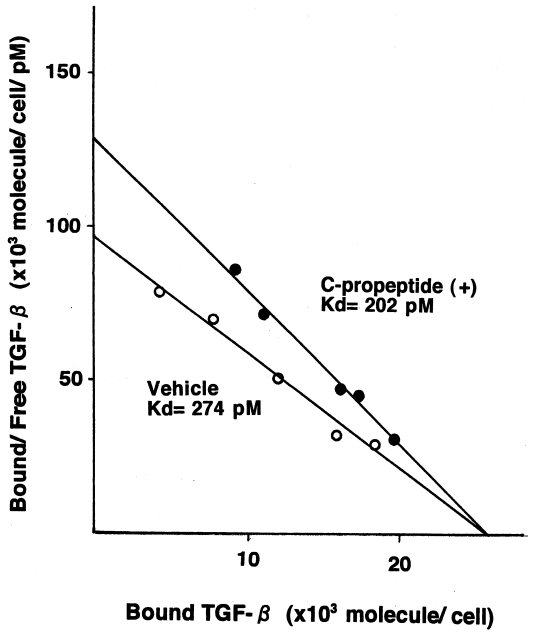

Fig. 2. Scatchard plot analysis of ^{125}I-labeled TGF-β binding to MC3T3-E1 osteoblastic cells. 2.5×10^5 cells that were cultured for 5 days after confluence were cultured for 1 h at 4°C in the presence of various concentrations of ^{125}I-labeled TGF-β. Each data point represents the mean of duplicate determination, and the experiments were repeated twice.

3.3. The affinity of TGF-β to its receptors after treatment with c-propeptide

The action of TGF-β is expressed by the interaction of TGF-β with its receptor on the cell membrane. There are three types of receptors, Type I, Type II and Betaglycan [30,31]. The affinity of TGF-β to its receptors is reduced after long-term cultures of MC3T3-E1 osteoblastic cell [11]. Therefore we investigated the affinity of TGF-β to receptors in the presence of c-propeptide by Scatchard analysis, and found that preosteoblasts cultured with c-propeptide (40 nM), showed higher affinity to TGF-β than cells treated with the vehicle (Fig. 2). These findings indicated that the action of TGF-β was enhanced in the presence of c-propeptide in preosteoblasts by the increased affinity of TGF-β to its receptor.

4. Discussion

The c-propeptide is a major secretory protein of osteoblasts [12]. It has been postulated that c-propeptide suppresses collagen synthesis in a feedback manner. In IMR-90 fibroblasts, 40 nM c-propeptide causes 80% reduction of collagen synthesis, while the synthesis of non-collagenous proteins is not affected [32]. Our result that c-propeptide suppressed collagen synthesis was consistent with previous findings. Furthermore, c-propeptide suppressed ALP activity dose dependently in preosteoblasts. This enzyme inhibition may have been due to the suppression of collagen synthesis, because ALP activity is markedly suppressed when either eliminating ascorbic acid or adding a collagen-synthesis inhibitor that inhibits collagen synthesis [33]. However, we could not exclude the possibility that the suppression of ALP activity by c-propeptide may be due to the change of culture conditions. Therefore, further investigation is necessary for clearing the mechanism of the effect of c-propeptide on ALP activity.

In mature osteoblasts, c-propeptide influenced neither collagen synthesis nor ALP activity. In another study, we have found that the insensitivity to c-propeptide in mature osteoblasts was due to a decrease of the interaction of c-propeptide with α2β 1 integrin receptor, which is a main receptor for c-propeptide (manuscript in preparation). The reduced interaction may be due to a decrease of receptor number, because Lynch et al. reported that β 1 integrin receptor gene expression is downregulated in a time dependent manner during the osteoblastic differentiation [34]. Another possibility for the lower sensitivity in osteoblasts might be the competition of type I collagen and c-propeptide for α2β 1 integrin receptors. Collagen and c-propeptide are produced equimolarly during collagen synthesis. Collagen shows a 20-fold higher affinity to α2β 1 integrin receptors than c-propeptide [35]. Therefore, collagen preferentially binds α2β 1 integrin receptors when collagen and c-propeptide are present simultaneously, and this competition may reduce the sensitivity of c-propeptide to osteoblasts.

TGF-β is one of the abundant growth factors present in bone matrix, and it is likely that TGF-β has a crucial role in linking bone formation and bone resorption [36]. On the other hand, TGF-β has been reported to inhibit ALP activity in MC3T3-E1 osteoblastic cells [9,29]. These findings are consistent with our results. TGF-β (2ng/ml) suppressed ALP activity in preosteoblasts; however, the inhibition was weaker in osteoblasts. This difference might be due to the reduction of accessibility of TGF-β to its receptor [11]. Takeuchi et al. found that the expression of type II TGF-β receptor, which is a main receptor in osteoblasts, is not reduced in osteoblastic differentiation, and the lesser accessibility might be due to (1) matrix proteins binding TGF-β, or (2) matrix proteins masking TGF-β receptors [11].

Next we investigated the effect of c-propeptide on the action of TGF-β on ALP activity in preosteoblasts. c-Propeptide additively inhibited ALP activity and the inhibition of enzyme activity by the c-propeptide increased in a dose dependent manner. These effects of c-propeptide may be due to the suppression of collagen synthesis. c-Propeptide binds α2β 1 integrin receptors and inhibits collagen synthesis in a pretranscriptional or posttranscriptional manner [19,20,35]. Collagen synthesis and osteoblastic differentiation are tightly correlated, because the inhibition of collagen synthesis suppresses osteoblastic differentiation [22,23,33]. Furthermore, type I col-

Table 2
The neutralization of the effect of c-propeptide by the anti c-propeptide polyclonal antibody

	TGF-β (ng/ml)	c-Propeptide (nM)	Anti c-propeptide antibody	ALP activity (IU/mg DNA)
Preosteoblast (5 days culture after confluence)	2	0	−	18.8 ± 2.3
	2	40	−	12.3 ± 2.8
	2	40	+	20.9 ± 3.7

Values are evaluated from four dishes and shown as mean ± S.D.

lagen supports the expression of osteoblastic phenotypes [34]. Therefore, c-propeptide might retard the osteoblastic differentiation of preosteoblasts, which was shown as the suppression of ALP activity. The cells treated with c-propeptide showed higher affinity to TGF-β than cells treated with the vehicle (Fig. 2), and this higher affinity may cause the higher inhibition of ALP activity by TGF-β. These findings imply that c-propeptide–integrin interaction may alter the affinity of TGF-β to its receptor in preosteoblasts.

On the other hand, there is a possibility that the inhibition of ALP activity by TGF-β and c-propeptide are independent phenomena. However, analysis of the binding of TGF-β to its receptor in our study implied that the binding of TGF-β to cells was influenced by c-propeptide–integrin interaction.

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