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Osteomodulin regulates diameter and alters shape of collagen fibrils

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ABSTRACT

Osteomodulin (OMD) is a member of the small leucine-rich repeat proteoglycan family, which is involved in the organization of the extracellular matrix. OMD is located in bone tissue and is reportedly important for bone mineralization. However, the details of OMD function in bone formation are poorly understood. Using the baculovirus expression system, we produced recombinant human OMD and analyzed its interaction with type I collagen, which is abundant in bone. In this result, OMD directly interacted with purified immobilized collagen and OMD suppressed collagen fibril formation in a turbidity assay. Morphological analysis of collagen in the presence or absence of OMD demonstrated that OMD reduces the diameter and changes the shape of collagen fibrils. We conclude that OMD regulates the extracellular matrix during bone formation.

1. Introduction

Extracellular matrix (ECM) not only contributes to the physical properties of all tissues but also regulates cell behavior, including proliferation and migration. The components of ECM such as collagen, fibronectin, and proteoglycans interact with each other and form tissue-specific structures [1,2]. Collagen, the most abundant protein in humans, is essential for tissue organization and maintenance. Type I collagen is the main component of type I-rich tissues such as skin and bone. It self-assembles, and collagen fibrils contribute to tissue strength. Collagen fibrils vary in thickness and shape, although these parameters are strictly regulated in each tissue [3]. For instance, bones have a unique collagen fibril structure, and fibril diameter is considered to be a critical element that determines the mechanical strength of bone [4]. Fibril shape is related to the strength of collagen fibrils [5], but the details of its regulation at the molecular level are not yet clear.

Small leucine-rich proteoglycans (SLRPs) are a family of ECM proteins involved in the regulation of collagen fibrils [6]. One of them, decorin, is present in a variety of tissues and its knockout in mouse skin leads to collagen fibrils of irregular diameter and increased skin fragility [7]. Functional analysis of the role of SLRPs in the regulation of collagen fibrils would contribute to elucidation of collagen fibril regulation and tissue organization. Osteomodulin (OMD), another member of the SLRP family, is abundant in bone [8,9]. This fact and the ability of OMD to bind to hydroxyapatite, an important component of bone mineralization, suggest that the function of OMD is closely related to bone formation. OMD is modified with glycosaminoglycans and its modification pattern changes in the course of bone development [10]. Yet, the function of OMD in bone is poorly understood.

The regulation of collagen fibrils is needed for bone formation [11]. In this study, we investigated the function of OMD in the formation of collagen fibrils. We expressed recombinant OMD in insect cells and analyzed its interaction with type I collagen and the effect of OMD on collagen fibrils. We found that OMD reduced the diameter and altered the shape of collagen fibrils. This new insight might lead to the elucidation of the molecular mechanisms of collagen fibril organization in the presence of OMD and the role of OMD in bone formation.

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2. Materials and methods

2.1. Expression and purification of OMD

Full-length human OMD cDNA encoding C-terminally FLAG tag was inserted into the pFASTBac1™ vector (Invitrogen). An extraction of bacmids was performed according to manufacturer's protocol (Bac-to-Bac Baculovirus Expression System, Invitorgen). After transfection of OMD bacmids into insect cells, they were incubated at 27 °C for 4 days; then the supernatant was passage 1 (P1) virus. Insect cells (Sf9; 1.0×10^6 cells/ml) were infected with P1 virus (1:25, v/v) and incubated with shaking at 125 rpm at 27 °C for 2 days. The supernatant was passage 2 (P2) virus. The above procedure was repeated to prepare P3 virus. For OMD expression, Sf9 cells (1.8×10^6 cells/ml) were infected with P3 virus (1:1000, v/v) and incubated with shaking at 125 rpm at 27 °C for 3 days.

OMD was purified from the supernatant by using anti-FLAG M2 affinity gel (Sigma–Aldrich) according to manufacturer's protocol. Bound protein was eluted with 1 M arginine-HCl (pH 4.4) and the eluate was immediately neutralized with 2 M Tris–HCl (pH 8.0).

OMD was further purified by size exclusion chromatography on a 26/60 Superdex 200 column (GE Healthcare). The running buffer contained 20 mM Tris–HCl (pH 8.0), 300 mM NaCl, and 400 mM arginine-HCl. Final purification was carried out on an analytical 10/300 Superdex 200 column. Phosphate-buffered saline (PBS, pH 7.4) was used as the running buffer. Purified OMD was detected by 12.5% SDS-PAGE and Western blotting with anti-DDDDK-tag mAb-HRP-DirecT (MBL).

2.2. Circular dichroism spectroscopy

Circular dichroism (CD) spectra of OMD (3.0 μM in PBS, pH 7.4) were recorded on a JASCO J-820 spectropolarimeter in a 1 mm path-length quartz cell at 25 °C. The spectra were averaged over 5 scans and the background (PBS alone) was subtracted. CD analysis is based on manufacture's protocol (JASCO Co.). Raw CD values were converted to mean residue ellipticity (MRE) as follows:

$$\mathrm{MRE} = \mathrm{ellipticity(mdeg)}/10 \times \mathrm{Cr} \times \mathrm{path\ \ length\ (cm)},$$

where Cr = residues × protein's concentration (mol/l).

2.3. Differential scanning calorimetry

Differential scanning calorimetry (DSC) measurements were performed on an automated VP-DSC microcalorimeter (VP-Capillary DSC, GE Healthcare). The DSC cell was heated from 10 °C to 100 °C at a rate of 60 °C h^{-1}. The concentration of OMD was 3.5 μM in PBS (pH 7.4). Data were analyzed by using a two-state model with ORIGIN7 software.

2.4. Solid-phase collagen binding assay

Purified human type I collagen (3.1 mg/ml, VitroCol, Advanced BioMatrix) was diluted with 0.01 M HCl to 10 μg/ml and used to coat 96-well plates for 16 h at 4 °C. Wells were washed with saline-Tween (0.9% NaCl, 0.05% Tween 20) and blocked with 5% skim milk for 1 h at room temperature. Wells were washed three times with saline-Tween. OMD was then added and incubated for 4 h at 37 °C. Wells were washed three times with saline-Tween, and anti-FLAG antibody (Anti-DDDDK-tag mAb-HRP-DirecT, MBL) diluted 1:5000 in PBS (pH.7.4) was added and incubated for 1 h at room temperature. Wells were washed three times with saline-Tween and once with saline. The signal was detected with 3, 3′, 5, 5′-tetramethylbenzidine (ScyTek Laboratories, Inc.). The reaction was stopped by adding stop solution (ScyTek Laboratories, Inc.). Absorbance was measured at 450 nm.

2.5. Turbidity assay

Turbidity assays were carried out according to [12]. Purified human type I collagen (3.1 mg/ml, VitroCol, Advanced BioMatrix) was dialyzed against PBS (pH 7.4) for 16 h at 4 °C. The final collagen concentration was 230 μg/ml and OMD was added at 0, 2.5, 5, 10, 20, or 40 μM. The solutions were incubated at 37 °C for 12 h in a JASCO 700 spectrophotometer. Absorbance was continuously measured at 400 nm.

2.6. Transmission electron microscopy

Samples were used after turbidity assay with or without 10 μM of OMD. Negative staining and transmission electron microscopy (TEM) were performed as previously described [13]. A Hitachi H-7500 electron microscope was used. Collagen diameter was measured with AxioVision Rel. 4.8 (Carl Zeiss) as previously described [14]. At least 45 samples per condition (with or without OMD) were analyzed. An *F*-test was carried out to investigate the difference in the distribution of fibril diameter between samples with OMD and those without OMD. A Welch's *t*-test was performed to investigate the differences in the mean diameter at each condition.

2.7. Scanning electron microscopy

Scanning electron microscopy (SEM) was performed with a Zeiss SIGMA microscope. Extra high tension was 2 kV. For each condition (with or without 10 μM OMD from turbidity assay), at least 3 images of collagen fibrils were collected in different regions.

3. Results

3.1. OMD preparation and physicochemical analysis

C-terminally FLAG-tagged recombinant human OMD (Fig. 1A) was produced in Sf9 cells and purified by affinity column chromatography and size exclusion chromatography. The elution profile of the purified protein showed a single peak (Fig. 1B). Coomassie staining of SDS-PAGE gels and Western blotting indicated that OMD was present in this peak (Fig. 1C). The yield was 0.6 mg/L of Sf9 cell culture.

CD analysis showed a negative peak at 218 nm (Fig. 1D), indicating the presence of beta-sheet structures. The DSC profile demonstrated that the thermal stability (T_m) of OMD was 52 °C and the temperature of the onset of denaturation was approximately 45 °C (Fig. 1E).

3.2. Solid-phase collagen binding assay

We assessed the binding of OMD to collagen immobilized on 96-well plates. OMD binding was observed in the presence but not in the absence of collagen (Fig. 2A). OMD bound to collagen in a concentration-dependent manner. Therefore, OMD is a novel protein binding to type I collagen.

3.3. Turbidity assay

Turbidity assay was carried out to investigate how OMD affects the formation of collagen fibrils (Fig. 2B). The turbidity of collagen fibrils increases as they grow and it can be monitored as increasing absorbance at 400 nm [12]. OMD decreased the rate of formation of

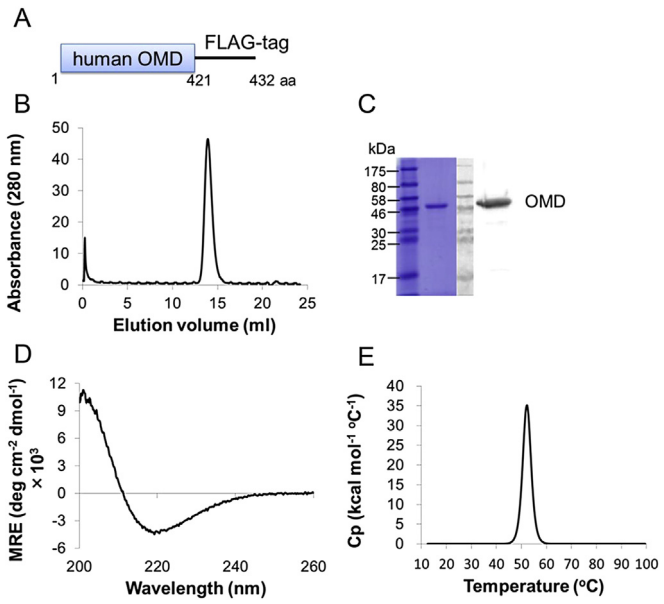

Fig. 1. Purification and physicochemical properties of recombinant human osteomodulin (OMD) produced in insect cells. (A) FLAG-tagged recombinant human OMD. (B) Purification of OMD by size exclusion chromatography. (C) SDS-PAGE (12.5%) and Western blot analysis of purified OMD. (D) Circular dichroism spectrum of OMD. MRE is mean residues ellipticity. (E) Thermal stability of OMD assessed by differential scanning calorimetry. Cp means specific heat at constant pressure.

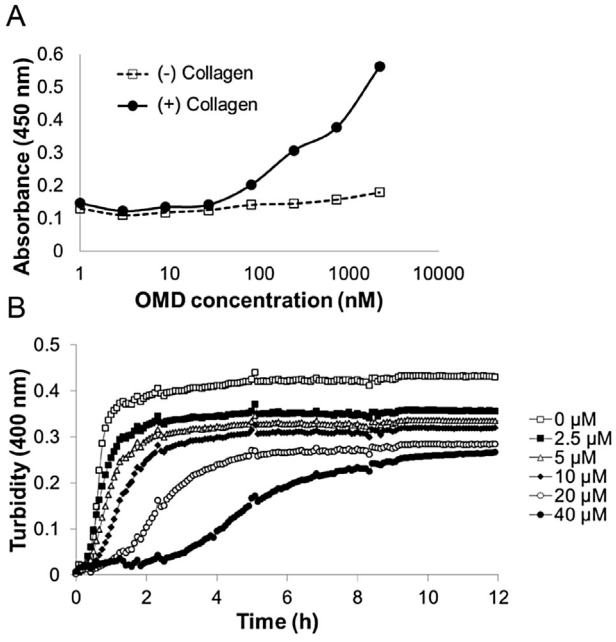

Fig. 2. Interaction of OMD with collagen. (A) Solid-phase assay of collagen binding to OMD. Wells of 96-well plates were coated or not (see figure key) with acid-solubilized collagen, blocked with skim milk and incubated with different concentrations of OMD, and then incubated with anti-FLAG antibody conjugated with horseradish peroxidase. Binding was detected as absorbance at 450 nm. (B) Turbidity assay. Acid-solubilized collagen was dialyzed against phosphate-buffered saline and incubated at 37 °C in the presence of different concentrations of OMD. Binding was detected as turbidity at 400 nm.

collagen fibrils in a concentration-dependent manner. The final turbidity at plateau also decreased with increasing OMD concentrations. Therefore, OMD suppressed both the rate and extent of collagen fibril formation.

3.4. TEM analysis

Collagen fibrils are formed by specific self-aggregation [3]. Collagen fibrils were visualized by TEM with negative staining. Collagen triple helices are aligned in a staggered manner; thus, the fibrils form overlap and hole zones, a pattern called D-periodicity [3]. In our TEM analysis, fibrils showed typical D-periodicity with overlap zones (black areas) and hole zones (gray areas) (Fig. 3A, B).

We used TEM imaging to investigate how OMD affects the morphology of collagen fibrils. We measured the fibril diameter in the absence or presence of OMD (Fig. 3C, D) In the presence of OMD, the diameter was 75 ± 15.7 nm (mean ± SD, standard deviation), whereas it was 94 ± 29.4 nm in the absence of OMD. The *P*-value (4.9×10^{-6}; *F*-test) indicated that the difference between distributions of the collagen fibril diameter was statistically significant. Therefore, the Welch's *t*-test (heteroscedastic *t*-test) was carried out. The *P*-value (3.3×10^{-4}) showed that the mean diameter in the presence of OMD was smaller than that in the absence of OMD. These results suggest that OMD reduces the collagen fibril diameter and its variability. Only a small number of collagen fibrils had a twisted shape in the presence of OMD, whereas approximately half of the fibrils were twisted without OMD (Fig. 3A, B, twist: white arrow, straight: black arrow).

3.5. SEM analysis

We used SEM to clearly visualize the 3D structures of collagen fibrils. As expected, the fibrils showed collagen-specific D-spacing (Fig. 4A, B), similar to the pattern in the TEM images (Fig. 3A, B). In the absence of OMD, most of the collagen fibrils formed twisted structures (Fig. 4A), whereas no twisted fibrils were observed in the presence of OMD (Fig. 4B) and some fibrils formed straight bundles. These results suggest that OMD suppresses the formation of twisted collagen fibrils.

4. Discussion

We found the presence of beta-sheets in the secondary structure of recombinant human OMD. Similarly, beta-sheets were reported

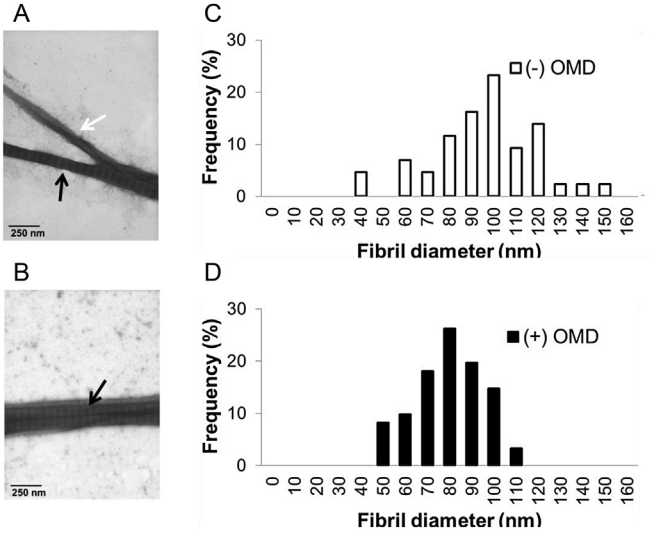

Fig. 3. Distribution of collagen diameter analyzed by transmission electron microscopy (TEM). Collagen fibrils were incubated at 37 °C for 12 h in (A, C) the absence or (B, D) the presence of 10 μM OMD. (A, B) Representative TEM images. (C, D) Frequency distribution of collagen diameter measured from TEM images.

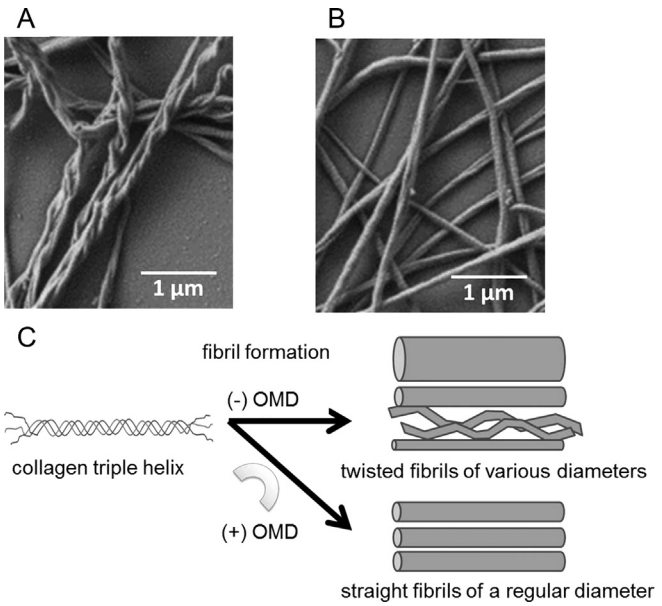

Fig. 4. Shape of collagen fibrils analyzed by scanning electron microscopy (SEM). (A, B) SEM images of collagen fibrils after incubation at 37 °C for 12 h in (A) the absence or (B) presence of 10 μM OMD. (C) A model of function of OMD in the regulation of diameter and shape of collagen fibrils.

in decorin [15]. The T_m onset of 45 °C indicates that OMD was stable in all our assays, which were performed at 37 °C.

We found that OMD binds to type I collagen. Other SLRPs (decorin, fibromodulin, and lumican) also directly bind to type I collagen [16,17]. Therefore, the interaction with collagen may be a common property of SLRPs. The binding signal was observed from 50 nM OMD and was not saturated at 2500 nM. Higher concentrations of OMD increased non-specific binding (data not shown). A recent study that used the same method also showed that decorin tends to non-specifically bind to collagen [18].

The ability of OMD to suppress the formation of collagen fibrils (as revealed by the turbidity assay) and to directly bind collagen suggests that OMD suppresses the interactions among individual collagen molecules to form fibrils. The change in turbidity is correlated with the lateral collagen fibril growth during fibril formation [19]. Therefore, OMD inhibits lateral fibril growth and consequently influences fibril morphology. Suppression of fibril formation has also been observed for decorin and some other SLRPs; in particular, decorin inhibits fibril formation [6].

We found that OMD decreases fibril diameter. In humans, the diameter of collagen fibrils is regulated differently in each tissue and it contributes to their mechanical strength [4]. Therefore, the regulation of fibril diameter is essential for tissue organization [20]. Decorin makes the fibrils thinner [21]. Fibromodulin also regulates collagen fibrils although its role in the direct regulation of fibril diameter has not been investigated [22]. The present study is the first to demonstrate that OMD reduces the diameter of collagen fibrils. Therefore, the regulation of collagen fibrils may be a common function of SLRPs. SLRPs are produced and function in a tissue-specific manner and may specifically regulate collagen fibrils in various tissues [3,23].

Collagen fibrils are generated by extensive branching and lateral fusion of collagen at a certain concentration [24]. Surprisingly, in the presence of OMD no twisted fibrils were observed, suggesting that OMD inhibits extensive lateral fusion among collagen fibrils because OMD perhaps covers on the surface of collagen fibril, resulting in straight fibrils. Although the role of SLRPs in the regulation of fibril diameter is discussed in the literature [6], little is known about their role in the regulation of fibril shape. Our results suggest that OMD can regulate both the diameter and shape of collagen fibrils (Fig. 4C). Further studies on the regulation of fibril shape are needed because the formation of collagen fibrils also depends on collagen concentration [24].

OMD is considered that it plays a role in bone mineralization [20]. To date, OMD has been reported to interact with alpha-v-beta-3 integrin [8] and bind to hydroxyapatite; the latter is a bone component. Our study revealed that OMD regulates the diameter and shape of collagen fibrils. In bones, the diameter and shape of collagen contribute to the mechanical strength [20]. Therefore, our study is important for understanding the potential role of OMD in the regulation of bone toughness and stiffness.

In conclusion, we demonstrated that OMD regulates the diameter and shape of collagen fibrils. This finding contributes to the elucidation of the SLRP function in bone formation.

Conflict of interest

None.

Acknowledgments

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