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Decorin modulates matrix mineralization in vitro

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Abstract

Decorin (DCN), a member of small leucine-rich proteoglycans, is known to modulate collagen fibrillogenesis. In order to investigate the potential roles of DCN in collagen matrix mineralization, several stable osteoblastic cell clones expressing higher (sense-DCN, S-DCN) and lower (antisense-DCN, As-DCN) levels of DCN were generated and the mineralized nodules formed by these clones were characterized. In comparison with control cells, the onset of mineralization by S-DCN clones was significantly delayed; whereas it was markedly accelerated and the number of mineralized nodules was significantly increased in As-DCN clones. The timing of mineralization was inversely correlated with the level of DCN synthesis. In these clones, the patterns of cell proliferation and differentiation appeared unaffected. These results suggest that DCN may act as an inhibitor of collagen matrix mineralization, thus modulating the timing of matrix mineralization.

Keywords: Osteoblast; Decorin; Collagen matrix mineralization

Mineralized tissues of vertebrates, except for tooth enamel, are essentially composed of two phases, i.e., mineral and fibrillar type I collagen, where collagen serves as a template to spatially organize mineralization. Although a number of non-collagenous matrix molecules have been proposed to modulate the timing of mineralization (i.e., initiation and inhibition), the mechanisms are still largely unknown.

Decorin (DCN), a member of small leucine-rich proteoglycans (SLRPs), is one of the well-characterized extracellular matrix molecules that regulate collagen fibrillogenesis [1]. It has been proposed that the concave surface of a horseshoe-shaped DCN molecule interacts with the triple helical domain of a single collagen molecule [2,3]. Several studies have demonstrated that DCN is removed or degraded prior to collagen matrix mineralization in vivo [4], suggesting its role in temporal regulation of mineralization. Although a targeted disruption of DCN gene alone does not reveal a major bone phenotype [5], when genes for DCN and its structurally related SLRP member, biglycan (BGN), are both disrupted, severe bone phenotypes, e.g., retarded bone growth and marked osteopenia, occur. These phenotypes are much more severe than those of the BGN single deficient animals [6], indicating a potential compensatory function of BGN for DCN.

In order to explore the potential roles of DCN in collagen matrix mineralization under more controlled conditions, osteoblastic cell clones expressing higher (sense-DCN, S-DCN) and lower (antisense-DCN, As-DCN) levels of DCN were generated, and the mineralized matrix formed by these clones was characterized.

Materials and methods

Cell cultures. The mouse calvaria MC3T3-E1 cells (subclone 26 [7], generous gift from Dr. Franceschi R.T., University of Michigan School of Medicine, MI) were maintained in α-minimum essential medium (α-MEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 atmosphere at 37 °C. The medium was changed twice a week.

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cDNA constructs and stable transfections. The cDNA sequence containing the coding region of the mouse DCN was isolated by reverse transcription-polymerase chain reaction (RT-PCR). The sequences of the primers were designed as follows: upper primer, 5′-GCGGATCCA TCATGAAGGCAACTCTCATC-3′ and lower primer, 5′-GCGCTCG AGCTTGTAGTTTCCAAGTTG-3′. The PCR product was then ligated into the pcDNA3.1/V5-His-TOPO mammalian expression vector (Invitrogen), sequenced at the UNC-CH DNA Sequencing Facility (University of North Carolina, Chapel Hill, NC), and sense- or antisense-orientated DCN containing plasmids were obtained. Each construct was transfected into MC3T3-E1 cells using FuGENE6 transfection reagent (Roche Diagnostics). After transfection, the cells were cultured and exposed to 400 μg/ml of G418 (Gibco) for 3–4 weeks to select stably transfected clones. Positive clones derived from single G418-resistant cells were then isolated by cloning rings and further grown in the same conditions. As a control, MC3T3-E1 cells were also transfected with an empty pcDNA3.1/V5-His A vector (Invitrogen) and subjected to the same selection and cloning procedures as described above.

Western blot analysis. To compare the DCN level among stable clones, Western blot analysis was performed using the polyclonal antibody against mouse DCN core protein, LF-113 [8] (generous gift from Dr. Fisher L.W., NIDCR, MD). Cells were washed with phosphate-buffered saline (PBS) twice. Cell/matrices were extracted with 6 M guanidine–HCl pH 7.4, dialyzed, and lyophilized. Equal amounts of dried samples were dissolved in sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris–HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, and 4% SDS) in the presence of 10 mM dithiothreitol (DTT), applied to 4–12% gradient SDS–polyacrylamide gel electrophoresis (PAGE), transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore), and reacted with LF-113. The DCN bands were immunostained with the Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad) and the levels were analyzed by Scion Image software (Scion Corporation).

Immunoprecipitation-Western blot assay. To verify that the DCN synthesized exogenously by S-DCN clones was a proteoglycan form, cultured media of both empty vector transfected clone (Ev) and S-DCN were immunoprecipitated with anti-V5 antibody (Invitrogen). After an addition of protein A–Sepharose 4B conjugate (Zymed Laboratories), the samples were incubated for an additional 30 min, and the beads were washed twice with the lysis buffer containing 150 mM NaCl, 20 mM Tris–HCl pH 7.5, 10 mM EDTA, 1% Triton X-100, 1% deoxycholate, 1.5% aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Proteins bound to the beads were treated or untreated with chondroitinase ABC (Seikagaku Corporation) at 37 °C for 2 h, dissolved in SDS sample buffer, and subjected to Western blot analysis with LF-113 as described above.

RT-PCR. Cells were plated at 2×10^5 cells/35 mm plastic dish (Falcon). When the cells reached confluency, the medium was replaced with the one supplemented with 50 μg/ml of ascorbic acid and 1 mM of β-glycerophosphate (mineralization medium), and maintained for the indicated days. Total RNA was extracted by means of the TRIzol Reagent (Invitrogen) solution. Two micrograms of the total RNA extract was used for RT using the Omniscript RT Kit (Qiagen) according to the manufacturer's protocol. PCR primers used in this study were designed based on the EMBL/GenBank (Accession No.: Runx2/Cbfa1, AF010284; Osterix, AF184902; GAPDH, M32599). After appropriate cycles of the reaction, aliquots of the PCR product were separated on 1.5% TAE–agarose gel, stained with ethidium bromide, and photographed under UV light. The intensity of each band was measured by Scion Image software.

In vitro mineralization assay. Cells were cultured as described above and maintained for up to 5 weeks. To detect the mineralized nodules, the samples were stained with Alizarin Red S (Sigma).

Results

Generation of cell clones expressing high (S-DCN) and low (As-DCN) levels of DCN

The results of the Western blot analyses showed that S-DCN clones synthesized higher (1.4–2.7-fold; Fig. 1A, lanes 3–5) and As-DCN lower (0.15–0.72-fold; Fig. 1A, lanes 6–8) levels of DCN in comparison with the control MC3T3-E1 cells as well as Ev clone (Fig. 1A, lanes 1and 2). When the DCN that is exogenously synthesized by S-DCN clones was immunoprecipitated with anti-V5 antibody and immunoreacted with anti-DCN antibody (LF-113), a broad band was detected at 66–97 kDa (Fig. 1B, lane 3), which corresponds to DCN proteoglycan found in the control MC3T3-E1 cells (Fig. 1A, lane 1).

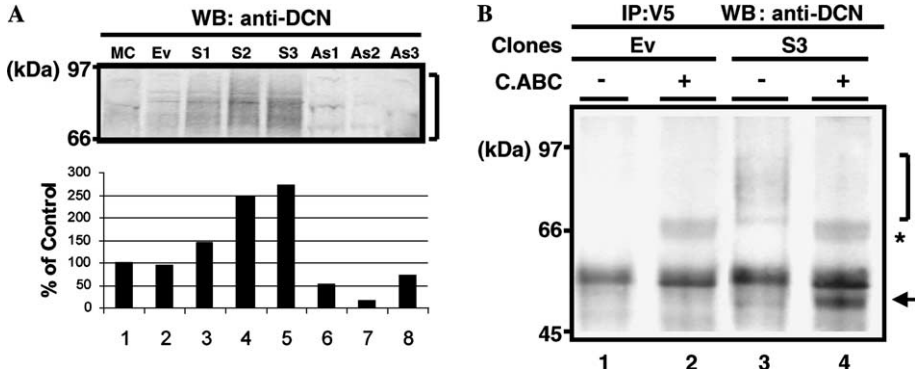

Fig. 1. Establishment of sense- and antisense-decorin clones. (A) Stable clones produce various levels of decorin protein. MC3T3-E1 osteoblastic cell lines were transfected with plasmid vectors containing DCN cDNA in sense (S)- or antisense (As)-orientation, or with an empty vector (Ev), and stable clones (S-DCN clones 1, 2, 3 → S1, S2, S3; As-DCN clones 1, 2, 3 → As1, As2, As3) were generated. Cell/matrices were extracted and analyzed by Western blotting (WB) with anti-DCN antibody (LF-113). The expression levels were measured and are shown by bars. Markers of molecular mass are shown on the left. (B) Overexpressed DCN protein is a proteoglycan. The cultured media from Ev and S-DCN (clone 3, S3) were immunoprecipitated (IP) with anti-V5 antibody (V5). The immunocomplex was treated or untreated with chondroitinase ABC (C.ABC) and detected by Western blotting with anti-DCN antibody (lanes 1–4). The proteoglycan is indicated by a bracket, the core protein is indicated by an arrow, and non-specific bands observed in C.ABC-treated lanes are indicated by an asterisk (*). Markers of molecular mass are shown on the left.

When treated with chondroitinase ABC, this band shifted to ~50 kDa as an immunoreactive band with LF-113 (Fig. 1B, lane 4). The broad band observed was also immunoreactive with anti-proteoglycan delta-Di-4S antibody, 2B6 (Seikagaku Corporation) (data not shown). These results confirmed that overexpressed DCN was a proteoglycan composed of a core protein and glycosaminoglycan (GAG), similar to the endogenous DCN in MC3T3-E1 cells.

Cell proliferation and the expression of Runx2/Cbfa1 and Osterix in stable clones

Since the overexpression of DCN may affect cell proliferation [9], the proliferation rates of S- and As-DCN clones were compared with those of control cells by performing cell counting and no difference was found (data not shown). To examine whether the manipulation of DCN protein could alter osteoblast differentiation, RT-PCR analyses with Runx2/Cbfa1 and Osterix primers, transcription factors essential for osteoblast differentiation [10,11], were conducted. The expression levels were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). As shown in Fig. 2, the expression levels of Runx2/Cbfa1 and Osterix were essentially the same among clones and control cells.

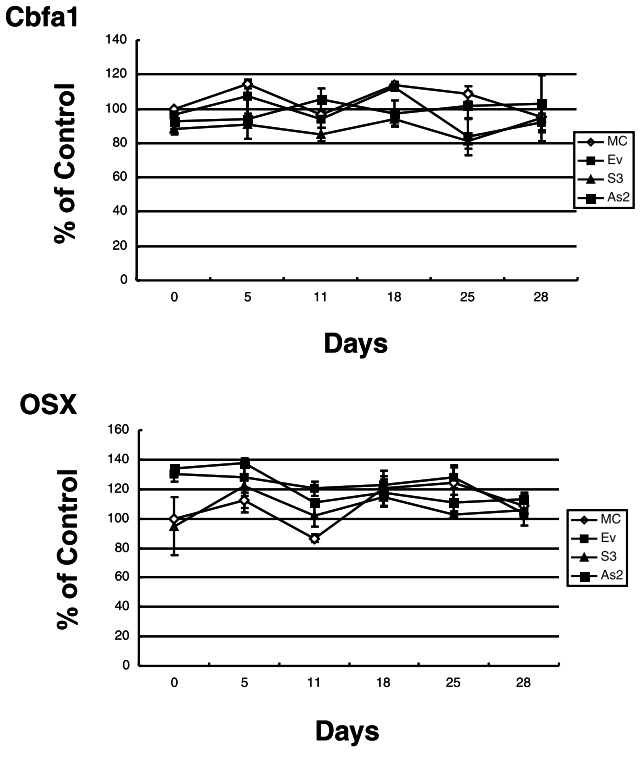

Fig. 2. mRNA expression profiles of osteogenic transcription factors. RT-PCR analyses with Runx2/Cbfa1 (Cbfa1) and Osterix (OSX)-specific primers were performed in three independent experiments. The intensity of each band was measured and normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The percentages indicate normalized expression levels and are shown as means ± SD.

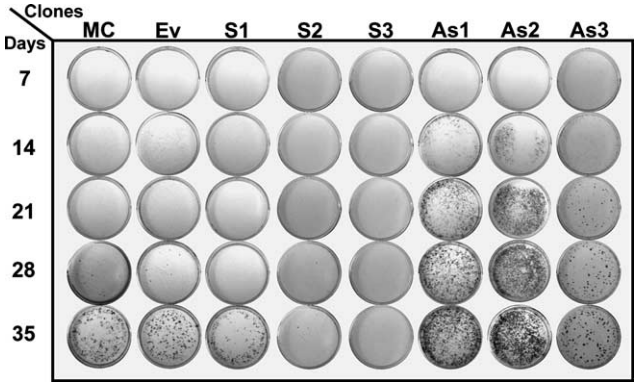

Fig. 3. Effects of DCN on mineralized nodule formation. MC3T3-E1 cells (MC), empty vector transfected cells (Ev), and S-DCN (S1, S2, and S3), and As-DCN (As1, As2, and As3) clones were plated in triplicate, cultured for up to 5 weeks and mineralized nodules were stained with Alizarin Red S. Three independent experiments produced similar results and one representative dish of each of all types at different time-points is shown.

Effects of DCN on matrix mineralization

Several S- and As-DCN clones along with controls (parental MC3T3-E1 cells and Ev clone) were cultured for up to 5 weeks after confluency and the mineralized nodules formed were stained with Alizarin Red S (Fig. 3). The mineralization pattern (e.g., timing and extent of mineralization) of the Ev clone was essentially identical to that of the parental MC3T3-E1 cells. The onset of mineralization in all S-DCN clones was significantly delayed when compared with controls. The delayed timing of mineralization in S-DCN clones was proportional to the DCN level synthesized. In contrast, the timing of mineralization in As-DCN clones was markedly accelerated and the number of mineralized nodules formed was significantly increased. The accelerated timing of mineralization was proportional to the level of suppression of DCN synthesis.

Discussion

MC3T3-E1 cells are well-characterized cell lines, which possess preosteoblastic phenotypes, and can form mineralized nodules after they differentiate into functional osteoblasts [12,13]. Using this cell line, stable clones that synthesize higher (S-DCN) and lower (As-DCN) levels of DCN were successfully established. The patterns of cell proliferation and differentiation were apparently not affected in these clones (see Results), indicating that the phenotypes observed in the clones were not due to the alteration of cell proliferation/differentiation.

All S-DCN clones examined exhibited significant delays in mineralization in comparison with the control MC3T3-E1 cells or Ev clone. The S3 clone which showed the highest DCN level (2.7-fold) did not form mineralized nodules even after 35 days in culture; in contrast, the onset of mineralization in all As-DCN clones was markedly accelerated, and in some clones the mineralized nodules could be detected on day 14, while they were detected after 3–4 weeks in control cells (Fig. 3). Thus, DCN apparently controls the timing of mineralized nodule formation in the mammalian cell culture system used in the present study.

Recent studies on DCN/BGN-double deficient mice have indicated that there is compensation of DCN by BGN in bone development [6,14]. In the present study, however, the mRNA expression level of BGN was unchanged among the clones and control cells (data not shown). This suggests, though needing to be confirmed by other assays, that in this culture system, a compensatory function of BGN for DCN is not significant at least during the culture periods conducted. Thus, this in vitro system may offer a useful model to investigate the functions of DCN in the process of matrix mineralization.

It is not clear at this point as to how DCN inhibits matrix mineralization in vitro. Since DCN binds to collagen via the leucine-rich repeats domain present in the protein core, the GAG component is readily exposed at the surface of collagen fibrils. This extended GAG, possibly with partially exposed protein core, may inhibit hydroxyapatite growth in and around collagen fibrils [15,16]. Alternatively, the inhibition may occur by regulating the assembly and stability of collagen fibrils [17], the template for mineralization, or through its interaction with other modulatory matrix molecules. Further studies are clearly warranted in order to elucidate the inhibitory mechanisms.

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