The modulation of collagen fibril assembly and its structure by decorin: An electron microscopic study*

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Summary. The present study was carried out to determine the effect of decorin in the process of collagen assembly. Collagen fibrils were obtained *in vitro* by aggregation from commercialized acid-soluble type I collagen with the addition of different concentrations of decorin (0–25 µg/ml). All specimens were observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The distribution of collagen fibril diameters was also analyzed by TEM. In samples without or with low concentrations of decorin, highly porous collagen fiber networks were formed. On the other hand, dense networks were observed in samples treated with high concentrations of decorin. The influence of decorin secreted by cells on collagen fibrils was observed by SEM, and the fiber network elasticity was measured using a rheometer. SEM images showed that collagen fiber networks without fibroblasts were much looser than those cultured with normal fibroblasts. The networks cultured with the

fibroblasts were composed of straight fibers with large diameters. On the other hand, collagen fiber networks cultured with siRNA-decorin-transfected (siDT) fibroblasts had loose, meandering fibers with small diameters. The elasticity of collagen fiber networks cultured with untransfected fibroblasts showed no significant difference over the 7-day incubation period. However, significantly lower elastic values were obtained for collagen fiber networks treated with siDT cells on days 3 and 7. In addition, after treatment with 5.0 or 25 µg/ml decorin, the collagen fiber networks cultured with siDT cells exhibited an altered structure that showed a dense structure similar to that of the fiber networks cultured with untransfected fibroblasts. In conclusion, this *in vitro* study showed that decorin is a regulatory and architecturally small leucine-rich repeat proteoglycan in the process of collagen fibril assembly.

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Introduction

Connective tissues, such as the dermis, bone, tendons and ligaments, are primarily composed of collagen fibers, which are formed by type I collagen. There are also various non-collagenous proteins — proteoglycans (PGs), and glycosaminoglycans (GAGs) — which interact with the fibrillar collagen network. Many studies have suggested that interactions between collagens, PGs, and/or GAGs play an important role in the process of collagen fibrogenesis and assembly.

Decorin, a small leucine-rich repeat PG (SLRP), is expressed by a variety of cells, including fibroblasts, myocytes, and smooth muscle cells (Thiesen and

Roesenquist, 1995; Hocking *et al.*, 1998, Nishimura *et al.*, 2003). In connective tissue, it is thought that decorin participates in the regulation of collagen fibrogenesis by binding collagen fibrils (Bidanset *et al.*, 1992; Kresse *et al.*, 1997; Iozzo *et al.*, 1999) in cooperation with other SLRPs and a variety of growth factors (Yamaguchi *et al.*, 1990; Hildebrand *et al.*, 1994; Grinnell *et al.*, 2000; Hosaka *et al.*, 2005). Sini *et al.* (1997) performed a morphological study that showed the role of decorin in the *in vitro* fibrogenesis of type I collagen and concluded that decorin exerted different effects on phases of fibril formation in correlation with the degree of glycosylation of the collagen. Therefore, decorin might influence the assembly and diameter of collagen fibrils by binding to their surfaces.

Several independent studies using materials such as chicken embryos and mouse tendons have suggested that decorin delays individual collagen molecule fusion (Vogel *et al.*, 1984; Vogel and Trotter, 1987; Birk *et al.*, 1995, 1996). It has also been shown that decorin determines the interfibrillar space of collagen fibrils by regulating the length of side chains (Kuwaba *et al.*, 2001, 2002; Watanabe *et al.*, 2005, 2007). Decorin also acts as an intermediary between type I collagen and some types of collagen (Nareyeck *et al.*, 2004).

Gene-targeting studies using decorin-deficient mice have shown that SLRPs are involved in the determination of the structural phenotype of collagen fibrils and connective tissue function (Danielson *et al.*, 1997). Decorin-deficient mice have fragile skin characterized by markedly reduced tensile strength and a thinner dermis than normal (Danielson *et al.*, 1997; Zhang *et al.*, 2006). Decorin deficiency also causes variation in collagen diameters *in vivo*, changing the mean and distribution profile of fiber diameters. Collagen alterations are observed throughout the connective tissue in decorin knockouts. Thus, decorin-knockout animals are useful for investigating decorin function in the whole body, though there are technical limitations in revealing the molecular event and mechanism of decorin in collagen fibrogenesis and its detailed assembly process *in vivo*. Despite these reports, much of the relationship between decorin and collagen structure has remained unknown. Especially, the relationship between decorin concentration and collagen fibrogenesis has not been fully determined.

In this study, we investigated the effect of decorin-collagen fibril interaction on collagen fibril assembly and its structure using type I collagen gel, decorin, and siRNA-decorin-transfected (siDT) cells. Collagen fibrils with or without decorin were examined by electron microscopy to determine how they were affected by treatment with decorin. The level of elasticity of collagen

gels was measured to evaluate their mechanical strength, and the relationship between their structure and this property was discussed.

Materials and Methods

The procedures used in the experiments are summarized in Figure 1.

Preparation of type I collagen gels

A commercially available kit (Cellmatrix type I-A, Nitta Gelatin, Osaka) of acid-soluble type I collagen gels was used for the reconstruction of collagen fibrils. This kit includes acid-soluble type I collagen, a 10 × minimum essential medium (MEM) and a reconstitution buffer (50 mM NaOH, 250 mM NaHCO₃ and 200 mM HEPES). Type I collagen gels were prepared according to the manufacturer's protocols. Briefly, Cellmatrix Type I-A (8 volumes) and 10 × MEM (1 volume) were mixed at 4 °C and then the reconstitution buffer (1 volume) was added. Various concentrations of decorin (0.1, 1.0, 5.0, 10 and 25 µg/ml; Sigma-Aldrich, St. Louis, MO, USA) were immediately added to the collagen gel mixture, and 1 ml of the solution was poured into each well of a 12-well plate. The reconstructed collagen gel mixture was allowed to set over a period of 30 min at 37°C. For the examination of gel contraction, some gels were released from the well wall and left to float in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml of penicillin and 100 µg/ml of streptomycin (Sigma-Aldrich) over a period of 7 days.

siRNA transfection

siRNA-decorin-transfected (siDT) cells were produced by suppressing the gene for decorin in mouse NIH3T3 fibroblasts using RNAi. siRNA was derived from the coding sequence of the mouse decorin gene (Genbank accession number: NM7833). siRNA selection was based on the program B-Bridge Japan (Tokyo). The target sequences and siRNA duplexes for these siRNAs were designated as siRNA (targeted sequence: 5'-AAG AGA GGC UUA UUU GAC UUC-3', sense: 5'-GAG AGG CUU AUU UGA CUU CdTdT-3', antisense: 5'-GAA GUC AAA UAA GCC UCU CdTdT-3').

Experiment design for decorin knockdown in NIH3T3 cells using siRNA

Two days before making the gel and cell mixture, 5×10^4 cells/well were plated in a six-well plate. The following day, one batch of the cells was transfected with GeneEraser (Stratagene, La Jolla, CA, USA) containing 10 nM siRNA in fetal bovine serum-free DMEM. The following day, siRNA-treated or untreated NIH3T3 cells were trypsinized and mixed with the gel solution (cell-gel mixture), and 1 ml of the mixture was poured into each well of a 12-well plate (final concentration: 5×10^4 cells/ml). Each well wall was coated with 3% poly-2-hydroxyethyl methacrylate (Sigma-Aldrich) in 95% ethanol to eliminate any adhesion of cells to the growth well surfaces. Some of the cell-gel mixture was poured into glass dishes (1 ml of mixture per 17 mm diameter dish) for rheological analyses. After the setting of the cell-gel mixture, the culture medium was added to the gels and changed every 3 days. The cell-gel mixture was sampled on days 15 and 27 after commencing culture in the three-dimensional environment. The cell-gel mixtures were also examined on days 1, 3, and 7 for gel elasticity. After the

setting of the cell-gel mixture, treatment with siRNA was performed every 6 days, and some groups were treated with 5.0 or 25 $\mu\text{g/ml}$ decorin instead of siRNA on day 18 and 24. Specimens were collected on day 27.

Sample preparation for ultrastructural analysis by scanning electron microscopy and transmission electron microscopy

Collected specimens were prepared for ultrastructural analyses by scanning electron microscopy (SEM) or transmission electron microscopy (TEM). After rinsing with a phosphate buffer, collagen gels were fixed in 3% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for 1 h at 4°C, and then samples were rinsed with distilled water, post-fixed in 1% osmium tetroxide for 1 h at room temperature, rinsed again with distilled water, and dehydrated in a graded concentration of ethanol. For SEM observation, the specimens were dried by the t-butyl alcohol freeze-drying method (Inoue and Osatake, 1988), mounted on metal stubs, coated with osmium using an osmium coater (Eiko IB3; Ibaraki), and observed

Fig. 1. Summary procedures used in the experimental procedures. Reconstructed collagen gel mixtures with various concentrations of decorin were incubated for 7 days and observed under an electron microscope (○). Specimens of cell-gel mixtures were sampled on days 1, 3, and 7 for measurement of the gel elasticity (●) and for SEM observation of the morphology of collagen fibrils (incubated for 15 days) (▲). SiRNA treatment demonstrated every 6 days and incubated for 27 days. In some specimens, 5.0 or 25 $\mu\text{g/ml}$ of decorin was added instead of siRNA from day 18 onwards. These specimens were collected for SEM observation (■).

under a scanning electron microscope (JSM-6000F, JEOL, Tokyo) with an accelerating voltage of 3 kV. For TEM observation, the samples were embedded in resin (Quetol 812). Ultra-thin (110 nm in thickness) sections were mounted on a copper grid, stained with 1% uranyl acetate for 8 min and 1% lead citrate for an additional 10 min, and examined by transmission electron microscope (JEM-1200, JEOL) with an accelerating voltage of 80 kV. For measurements of collagen fibril diameter and area of collagen fibrils per unit, 500 fibrils were randomly selected in photographs of each collagen gel. Independent experiments were carried out three times.

Rheological analysis and measurement of gel elasticity

A spherical plunger of 7 mm in diameter was stabbed into the gels at a rate of 0.5 mm/sec using a creep meter (RE-33005, Yamaden, Tokyo). The apparent elasticity of the collagen gels was calculated from the load versus indentation curve obtained using an equation based on Hertz's theory (Lee and Radok, 1960):

$$F = (16\sqrt{R}/3) \times G \times 1.5h,$$

where F is the load (N), R is the radius of the plunger (3.5 mm), G is apparent elasticity (Pa), and h is the indentation (0.7 mm).

Statistical analysis

One-way analysis of variance (ANOVA) followed by Fisher's test using Statview® for Windows, version 5.0, was applied for analyses of the area of collagen fibril per unit, and Student's t -test, Microsoft Office Excel 2003 applied rheological analysis. In both results, significant difference was set at $P < 0.05$.

Fig. 2. SEM images and histograms of the diameter of collagen fibrils in a collagen fiber network. Collagen fiber network treated with 0 (control, **a**), 0.1 µg/ml (**b**), 1.0 µg/ml (**c**), 5.0 µg/ml (**d**), 10 µg/ml (**e**), and 25 µg/ml (**f**) of decorin. Membranous structures (arrowheads) appear in the fiber network treated with low concentrations (0 and 0.1 µg/ml). Bar=1 µm. **g**: TEM images of collagen fibrils in a collagen fiber network treated with decorin. Bar=100 nm.

Results

Effects of decorin on collagen assembly and fibril diameter

Highly porous collagen fiber networks were formed without decorin (control) or with low concentrations of decorin (0.1 or 1.0 $\mu\text{g/ml}$) (Fig. 2a–c). Membranous structures were often observed in these fiber networks. In contrast, the addition of 5.0 $\mu\text{g/ml}$ decorin tightened the mesh network, and similar dense fiber networks were also produced in the samples treated with 10 or 25 $\mu\text{g/ml}$ decorin (Fig. 2d–f). Membranous structures were not found in these fiber networks. Furthermore, the gels treated with high concentrations of decorin were not released from the well walls, indicating that the gel did not contract in these samples. The unreleased gel was still adhered to the well walls at the end of the incubation period (day 7). Between the released gels and unreleased gels, collagen fibril assembly did not appear to differ at any concentration (data not shown).

The average collagen fibril diameter was unchanged regardless of the concentration of decorin, and the median collagen fibril diameter was similar in all specimens. However, the range of collagen fibril diameters in the fiber networks was smaller in the samples treated with a high concentration of decorin than in the samples treated with decorin at a concentration less than 1.0 $\mu\text{g/ml}$ (Fig. 2, Table 1). The densities of collagen fibrils (percentage to occupation rate of collagen fibrils in cross section fields) were obtained from TEM micrographs. These values in the fiber networks without decorin (control) and with decorin (0.1, 1.0, 5.0, 10, 25 $\mu\text{g/ml}$) were 9.0 ± 0.3 , 10.3 ± 1.3 , 9.6 ± 1.0 , 16.7 ± 1.0 , 16.3 ± 1.5 , and 16.5 ± 1.4 , respectively. Significantly high densities of collagen fibrils (%) were observed in the samples treated with 5.0 $\mu\text{g/ml}$ or more of decorin as compared with the density in controls (Fig. 2, Table 1).

Effect of decorin on collagen fibrils and fiber network elasticity

SEM images showed that collagen fiber networks without fibroblasts were much looser (Fig. 3a) than those cultured with untransfected fibroblasts (Fig. 3b). The networks cultured with untransfected fibroblasts were composed of almost straight fibers with large diameters. On the other hand, collagen fiber networks cultured with siDT fibroblasts were loose and composed of collagen fibers with small diameters (Fig. 3c). The elasticity of collagen fiber networks with untransfected fibroblasts showed no significant difference over the incubation period. In contrast, on days 3 and 7, significantly lower elastic values than that on day 1 were obtained for collagen fiber networks cultured with siDT fibroblasts (Fig. 3d).

Recovery of collagen fibril assembly by addition of decorin after decorin knockdown

Collagen fiber networks cultured with siDT fibroblasts (Fig. 4b) were much looser than networks cultured with untransfected fibroblasts (Fig. 4a). After collagen fiber networks cultured with siDT fibroblasts had been supplemented with 5.0 or 25 $\mu\text{g/ml}$ decorin, the structure of the fiber networks changed from loose to dense and became similar to the structure of fiber networks cultured with untransfected fibroblasts (Fig. 4c, d).

Discussion

By morphological and mechanical analyses, we found that decorin significantly influences collagen fibril assembly, diameter, and mechanical strength.

It is thought that decorin binds to the surface of collagen fibrils *via* its core protein and that it regulates collagen fibril morphology (Vogel and Trotter, 1987;

Table 1. Parameter of collagen fibrils

Decorin concentration ($\mu\text{g/ml}$)	0 (control)	0.1	1.0	5.0	10	25
Mean (nm)	11.8 ± 5.37	11.5 ± 5.42	11.5 ± 5.14	11.5 ± 4.44	11.8 ± 4.29	11.7 ± 4.71
Median (nm)	10.8	10.8	10.6	10.8	11.2	11.1
Range (nm)	2.62-35.7	2.62-35.9	3.18-31.9	2.94-28.9	3.17-26.3	3.16-26.8
Density of collagen fibrils in cross section fields (%)	8.95 ± 0.37^a	10.3 ± 1.33^a	9.63 ± 0.97^a	16.3 ± 1.09^b	16.3 ± 1.51^b	16.5 ± 1.15^b

Different letters (*a* and *b*) indicate significant differences ($P < 0.05$)

Fig. 3. SEM images of collagen fibrils on day 15 cultured without cells (gel only, **a**), with untransfected fibroblasts (**b**), and with siDT fibroblasts (**c**). Bar=1 μ m. Elasticity of the collagen fiber network cultured with untransfected fibroblasts and siDT fibroblasts (**d**). *: Significant difference against the value on day 1 in the same group ($P<0.05$).

Fig. 4. Effects of decorin on recovery procedure in collagen fibril assembly. All pictures were taken on day 27. Fiber networks cultured with untransfected fibroblasts (**a**) and siDT fibroblasts (**b**) and 5.0 (**c**) or 25 μ g/ml decorin (**d**)-treated fiber networks cultured with siDT fibroblasts. Bar=1 μ m. **Inset** in **a** is a micrograph of the specimen treated with only a transfection reagent without siRNA.

Bidanset *et al.*, 1992; Scott, 1996, 2001; Raspanti *et al.*, 2002). Decorin also plays a role in the assembly of collagen fibrils, being involved in the determination of fibril diameter (Danielson *et al.*, 1997; Corsi *et al.*, 2002). Our data support results of previous studies and further revealed that an increase in the amount of decorin in the process of collagen assembly results in an increase in density of the collagen fibril network. In addition, membranous structures could be seen in the

fiber networks either not treated or treated with very low concentrations of decorin. These findings suggest that decorin is an essential regulatory factor in the process of collagen aggregation.

Danielson *et al.* (1997) demonstrated that decorin-deficient animals exhibited a wider range of collagen fibril diameters than did wild-type specimens. However, our study clarified that decorin has indistinct effects in changing the average diameter of collagen fibrils. Our

study showed that the average diameter of collagen fibrils was not significantly changed regardless of the concentration of decorin. It is generally agreed that decorin is involved in fibril formation because it inhibits an increase in collagen fibril size *in vivo* (Weber *et al.*, 1996; Naeme *et al.*, 2000; Reed and Iozzo, 2002). Our previous studies demonstrated that the density and distribution of collagen fibril diameters in a tendon, which expresses high levels of type I collagen, showed regional differences that were concomitant to the amount of decorin (Watanabe *et al.*, 2005). However, it should be pointed out that various other factors might be involved in the regulation of collagen fibrogenesis *in vivo*, and further studies are needed to identify those factors.

Irregular profiles and abnormal fibril fusion patterns have been observed *in vivo* in decorin-deficient mice. These animals have extremely fragile skin that tears very easily (Danielson *et al.*, 1997; Zhang *et al.*, 2006). In our samples, the elasticity of collagen fiber networks treated with siDT fibroblasts showed significantly lower values. This might be caused by structural fragility due to a lack of decorin. Moreover, the addition of decorin (5.0 or 25 $\mu\text{g/ml}$) to the samples with siDT fibroblasts allowed collagen to adopt a normal fibril morphology. These results again indicate that decorin is the "architectural" SLRP that maintains collagen fibril configuration and provides strength of structure rheologically.

In the present study, we applied the RNAi method to control the expression of decorin in collagen fiber networks and observed drastic structural changes. Application of RNAi methods to other SLRPs may be useful for understanding the collagen fibrogenesis procedure more precisely. Moreover, suppression of decorin expression using RNAi may possibly serve toward the therapeutic treatment of some connective tissue disorders — such as scar formation — since decorin mRNA and protein are abundantly expressed and localized in scar connective tissue (Napoli *et al.*, 1990; Lee *et al.*, 2004). Thus, injection of decorin mRNA-suppressed cells (*via* decorin gene knockdown) into scar connective tissue may be useful to induce a softening of collagen tissues.

Our study was carried out three-dimensionally by SEM, whereas most previous studies were conducted two-dimensionally by TEM. As Raspanti *et al.* (2007) recently pointed out, TEM has a higher resolution and offers an excellent depiction of detailed collagen structures. We applied TEM to measure the diameter and density of collagen fibers; however, there are limitations in the thickness of the sections (around 100 nm). Under these conditions, the whole structure of the specimens above and below individual sections is difficult to determine unless serial sections are made. By using SEM, we can

observe in detail the whole structure of collagen fiber networks, thickness (diameter), and degree of aggregation, fusion, and orientation of collagen fibers.

In conclusion, this *in vitro* study showed that decorin is a key regulatory SLRP in the process of collagen fibril assembly and collagen morphogenesis. However, further, detailed investigation is required to determine the function of each SLRP (biglycan, lumican, and fibromodulin) in the complex process of collagen morphogenesis.

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