

Elongated dermatan sulphate in post-inflammatory healing skin distributes among collagen fibrils separated by enlarged interfibrillar gaps

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It has been reported that the disaccharide composition of dermatan sulphate shows transient changes after epicutaneous application of the hapten 2,4-dinitrofluorobenzene to mouse skin, and that these changes are most conspicuous in healing skin on day 15 after chemical insult [Kuwaba, Nomura, Irie and Koyama (1999) *J. Dermatol. Sci.* **19**, 23–30]. In the present study it was found that the molecular size of dermatan sulphate was increased on day 15 after hapten application. The molecular size of decorin increased in healing skin, whereas the size of dermatan-sulphate-depleted core protein did not increase. The length and localization of decorin dermatan sulphate were investigated by electron microscopy. Dermatan sulphate filaments oriented

orthogonally to collagen fibrils were longer in healing skin than in control skin. In control skin, dermatan sulphate filaments were found among tightly packed collagen fibrils. In contrast, the interfibrillar gaps between each collagen fibril were enlarged in healing skin; elongated dermatan sulphate filaments extended from the surface of collagen fibrils across the enlarged gap. These results suggest that the increase in molecular size of decorin dermatan sulphate is important in organizing collagen fibrils separated by enlarged interfibrillar gaps in healing skin.

Key words: Cupromeronic Blue, decorin, hapten.

INTRODUCTION

Dermatan sulphate (DS) is a glycosaminoglycan (GAG) of proteoglycans such as decorin or biglycan, which belongs to the group of small leucine-rich proteoglycans and contains a core protein substituted with one or two chondroitin sulphate and/or DS chains. DS consists of either glucuronic acid or iduronic acid alternating with *N*-acetylgalactosamine. DS reportedly changes in its extent and position of sulphate substitution and in the level of epimerization from glucuronic acid to iduronic acid, depending on age, differentiation and pathological conditions [1].

The dominant proteoglycan of dermis is decorin, which is regularly distributed at the surface of collagen fibrils *in situ* [2,3]. Decorin is involved in regulating the formation of collagen fibrils [4–10]. Binding of decorin to collagen is mediated by specific binding of its core protein because the DS-depleted core protein shows binding capacity [7]. Although DS is not essential for the binding of decorin to collagen, it has been proposed that DS controls the size of the interfibrillar space between collagen fibrils in the cornea and sclera of the eye [11]. Furthermore, DS promotes the inhibition of thrombin activity by heparin cofactor II [12]. It has been reported that DS binds hepatocyte growth factor or basic fibroblast growth factor [13,14]. These studies strongly suggest that the function of DS is closely associated with the organization of the extracellular matrix and the regulation of cell proliferation under normal physiological and even pathological conditions. However, their precise mechanisms remain unclear.

We have reported that the molar percentage of 4,5-unsaturated 2-*O*-sulpho-HexA-(β 1-3)-4-*O*-sulpho-GalNAc (Δ Di-diS_B) from DS decreased transiently in healing skin after epicutaneous application of the hapten 2,4-dinitrofluorobenzene (DNFB) to the dorsal skin of mice and that these changes were most conspicuous on day 15 after chemical insult, when early inflammatory responses had subsided and healing was proceeding [15].

In the present study we investigated the molecular size and distribution *in situ* of DS in healing skin. We found that the molecular size of decorin DS increased in healing skin and that elongated DS filaments in healing skin were distributed among collagen fibrils separated by enlarged interfibrillar gaps.

EXPERIMENTAL

Induction of dermatitis

Female Balb/cA mice (6–8 weeks) were purchased from Japan Clea (Tokyo, Japan). DNFB (Sigma, St. Louis, MO, U.S.A.) (10 μ l; 0.5%, v/v) in acetone was applied to four regions of the shaved dorsal skin. The control mice were treated with acetone alone [16]. Mice were killed by overanaesthesia on day 15; the dorsal skin was excised for the preparation of DS or proteoglycan and ultrastructural observations.

Preparation of DS

GAG was extracted as described previously [15] and suspended in 5% (w/v) calcium acetate/0.5 M acetic acid. DS was enriched from a GAG preparation by precipitation in 25% (v/v) ethanol. Cellulose acetate electrophoresis and disaccharide analyses were performed as described previously [15].

Molecular sieve chromatography

Molecular-mass distribution was analysed with HP 1050 Series HPLC Modules as hardware and Model LC100W/F PC workstation GPC version 1 software (Hewlett-Packard Company, U.S.A., Yokogawa Electric Corporation, Tokyo, Japan). The conditions were as follows: column, Shodex Ohpack SB804HQ-803HQ-8025HQ (8.0 mm \times 300 mm; Showa Denko, Tokyo, Japan); mobile phase, 0.2 M NaCl; flow rate, 0.8 ml/min;

Abbreviations used: DS, dermatan sulphate; DNFB, 2,4-dinitrofluorobenzene; GAG, glycosaminoglycan; TGF- β , transforming growth factor β .

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column temperature, 30 °C; detection, differential refractive. A calibration curve was first made by using pullulan with molecular masses of 5.8, 12.2, 23.7, 48, 100, 186, 380 and 1660 kDa. In accordance with the instruction manual for the software, the calibration curve was corrected with chondroitin sulphate from bovine nasal septa, which reportedly has a weight-average molecular mass of 19.8 kDa and a number-average molecular mass of 15.5 kDa [17,18].

Preparation of proteoglycan

Proteoglycan was prepared as follows. Skin samples on day 15 were minced and washed in 0.15 M NaCl/protase inhibitor solution containing 0.1 M 6-aminohexanoic acid, 10 mM EDTA and 5 mM benzamidine/HCl. Then samples were extracted with 8 M urea/0.05 M Tris/HCl (pH 7.4)/0.1 M NaCl containing protease inhibitors and shaken for 72 h at 4 °C. After centrifugation (15000 *g* for 30 min at 4 °C), the extract was gently mixed overnight with a gel suspension of DEAE-Toyopearl 650S in 8 M urea buffer containing the protease inhibitors at 4 °C. After standing, the non-adsorbed fraction was removed and gels were washed with 8 M urea buffer. The adsorbed fraction was eluted with 8 M urea/0.05 M Tris/HCl (pH 7.4)/3 M NaCl containing protease inhibitors. Proteoglycan was precipitated by the addition of 3.5 vol. of 95% (v/v) ethanol/1.3% potassium acetate, washed with 70% (v/v) ethanol and air-dried.

Electrophoresis and Western blotting

SDS/PAGE was performed in a gel containing 7.5% (w/v) polyacrylamide by the method of Laemmli [19]. After electrophoresis, gels were stained with 0.5% (w/v) Alcian Blue in 25% (v/v) ethanol/10% (v/v) acetic acid to reveal GAGs or were subjected to Western blotting. To detect a core protein of decorin, the proteoglycan fraction (approx. 1 µg as GAGs) was digested with 0.02 unit of protease-free chondroitinase ABC (EC 4.2.2.4) (Seikagaku Corp., Tokyo, Japan) at 37 °C for 1 h in 0.1 M Tris/HCl (pH 8.0) containing 0.03 M sodium acetate and protease inhibitors. Samples were then boiled for 5 min with SDS sample buffer containing 2-mercapethanol. The aliquot was subjected to SDS/PAGE in a gel containing 12.5% (w/v) polyacrylamide followed by Western blotting. Samples in a gel were electrotransferred to a PVDF membrane (Millipore Corp., Bedford, MA, U.S.A.) with an electrophoretic transfer cell (Bio Craft, Tokyo, Japan) operating at 0.12 A overnight in an ice bath. After treatment with a blocking solution of 10% (w/v) skimmed milk in PBS/Tween for 1 h at room temperature, rabbit serum against the core protein of decorin [20] was left to react for 1 h at room temperature. The membrane was then washed and incubated with horseradish-peroxidase-conjugated goat anti-rabbit IgG (Cappel, Durham, NC, U.S.A.) for 1 h at room temperature and washed again. Bound antibodies were revealed with ECL[®] kit (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.). Prestained protein molecular-mass standard was purchased from Bio-Rad (Hercules, CA, U.S.A.).

Electron microscopy

One mouse in each of the acetone-treated and DNFB-treated groups was killed on day 15 by overanaesthesia; dorsal skin was cut into small pieces (approx. 1 mm × 1 mm). The skin samples were fixed immediately in 2.5% (w/v) glutaraldehyde/25 mM acetic acid /25 mM sodium acetate (pH 5.8) at 4 °C overnight, followed by washing. To examine DS, samples were stained with

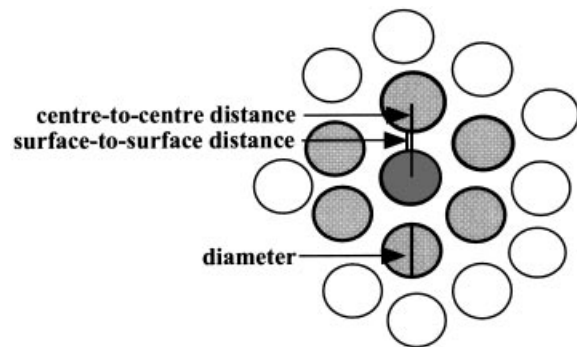


Figure 1 Measurement of fibril diameter and interfibrillar distance of collagen fibrils

A collagen fibril surrounded by five or six neighbouring fibrils was selected at random (dark grey); the distance between the centre of the central fibril and that of each surrounding fibril (light grey) was measured as the centre-to-centre distance. The length of the line between the surface of the central fibril and that of the surrounding fibril was also measured as the surface-to-surface distance.

0.05% (w/v) Cupromeronic Blue/0.1 M MgCl₂/25 mM sodium acetate buffer (pH 5.8)/2.5% glutaraldehyde (w/v) for 3 h at room temperature. The samples were washed, stained further with 0.034 M Na₂WO₄, dehydrated, embedded and cut into ultrathin sections (80–90 nm) [21]. The specimens were observed by electron microscopy without further electron staining. For observation of collagen fibrils, skin samples were fixed as described above, followed by post-fixation in 1% (w/v) OsO₄ in PBS, dehydration and embedding. The ultrathin sections were stained with uranyl acetate followed by lead citrate. All the electron microscopic observations were performed in the upper dermis just below the epidermis with the use of a Hitachi 7100 electron microscope (Hitachi, Tokyo, Japan) at a magnification of ×50000. The photographs were selected randomly from each of the acetone-treated and DNFB-treated groups and were scanned with Adobe Photoshop 5.0J on an Apple Macintosh computer. Cupromeronic Blue-stained filaments, which were oriented orthogonally to collagen fibrils, were chosen in each photograph of the longitudinal section of collagen fibrils; the lengths were measured with NIH image 1.61. Collagen fibril diameters were measured in uranyl acetate/lead citrate-stained cross sections (Figure 1). A collagen fibril that was surrounded by five or six neighbouring fibrils was selected, and the distance between the centre of the central fibril and that of each surrounding fibril (centre-to-centre distance) was measured with NIH image 1.61. The distance between the surface of the central fibril and that of the surrounding fibril (surface-to-surface distance) was also measured. In both groups, 400 measurements each of the length of DS filaments, the fibril diameter, the centre-to-centre distance and the surface-to-surface distance were conducted; three independent experiments were performed.

Statistics

Data on length of DS filaments, fibril diameter, centre-to-centre distance and surface-to-surface distance from three independent experiments were combined and are presented as histograms with the median. The Mann–Whitney *U* test was used to assess the difference because the data did not show a Gaussian distribution. *P* < 0.05 was considered significant.

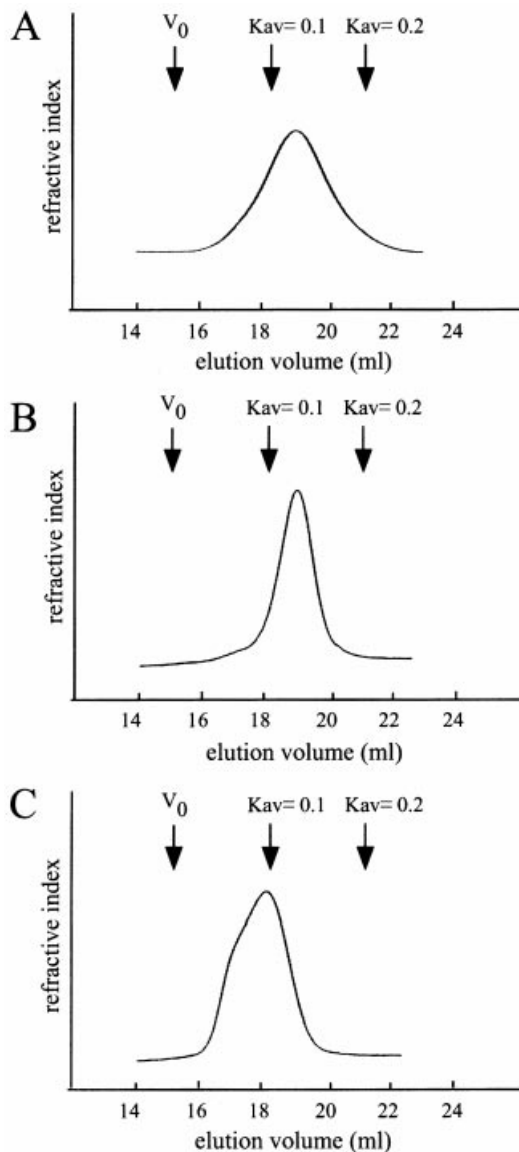


Figure 2 Molecular-mass distribution of DS

Elution patterns of molecular sieve chromatography of standard chondroitin sulphate used to correct a calibration curve made with pullulan (A) and the DS fraction in acetone-treated skin (B) and DNFB-treated skin (C) on day 15. The weight-average molecular masses of DS in acetone-treated skin and in DNFB-treated skin from three independent sets of experiments were 15.4 and 25.9 kDa, 16.3 and 32.2 kDa, and 18.2 and 29.6 kDa respectively. Representative data are shown. V_0 , 15.1 ml; total volume, 45.2 ml.

RESULTS

Increase in molecular size of DS in healing skin

On cellulose acetate electrophoresis of the DS-enriched fraction, only one spot stained with Alcian Blue was observed. This spot disappeared completely after treatment with chondroitinase ABC but not with chondroitinase ACII, indicating that the DS-enriched fraction was mostly composed of DS (results not shown). The disaccharide analysis of the DS-enriched fraction showed that hyaluronan-derived unsaturated disaccharide constituted approx. 2% of the total amount of unsaturated disaccharides released by chondroitinase ABC.

Figure 2(A) shows an elution profile of the standard chondroitin sulphate from bovine nasal septa whose weight-average molecular mass was said to be 19.8 kDa [17]. DS in acetone-treated skin was eluted at a partition coefficient of 0.13 with the peak top molecular mass of 14.8 kDa (Figure 2B), whereas DS in DNFB-treated skin had a partition coefficient of 0.10 with the peak top molecular mass of 21.4 kDa (Figure 2C). The molecular-mass distribution of DS was broader in DNFB-treated skin than in acetone-treated skin; the distributions overlapped to some degree. The weight-average molecular mass was 15.4 kDa in acetone-treated skin and 25.9 kDa in DNFB-treated skin. The small amount of potentially contaminating protein-derived components and hyaluronan did not affect the data on the molecular size of DS, because protein-derived components (partition coefficient = 0.27 ± 0.01) were separated evidently from DS; the hyaluronan was eluted at the void volume. Thus it was clearly shown that the DS obtained from healing skin on day 15 after DNFB treatment was longer than that from control skin after treatment with acetone.

Molecular size of decorin

To examine the molecular size of decorin, we performed SDS/PAGE and Western blotting of the proteoglycan fraction by using specific antiserum against the core protein of decorin. Figures 3(A) and 3(B) show that the molecular size of decorin was larger and more heterogeneous in DNFB-treated skin than in acetone-treated skin, although they overlapped partly between the two experimental groups. This is consistent with the observation that the molecular-mass distribution of DS was larger and broader in DNFB-treated skin than in acetone-treated skin, with some overlapping region (Figure 2). In contrast, no difference between the molecular sizes of a core protein was observed (Figure 3C). These results show clearly that the molecular size of decorin DS increased in DNFB-treated skin during healing processes, although we do not exclude the possibility that the size of GAG chains of proteoglycans other than decorin might have been affected by DNFB treatment.

Distribution of DS among collagen fibrils *in situ*

Decorin binds at the surface of collagen fibrils [4,8]. To elucidate the relationship between the elongation of DS chain of decorin and the arrangement of collagen fibrils in healing skin, sizes of collagen fibrils and decorin DS were measured by electron microscopy.

Figures 4(A) and 4(C) show cross-sectional profiles of collagen fibrils stained with uranyl acetate and lead citrate. In DNFB-treated skin, thin collagen fibrils were packed loosely and heterogeneously. The diameter of the collagen fibrils in DNFB-treated skin (Figure 5B; median 41.8 nm) was significantly thinner than that in acetone-treated skin (Figure 5A; median 77.0 nm). The centre-to-centre distance between the collagen fibrils was also significantly smaller in DNFB-treated skin (Figure 5D; median 68.3 nm) than in acetone-treated skin (Figure 5C; median 89.0 nm). In contrast, the surface-to-surface distance between the collagen fibrils was significantly larger in DNFB-treated skin (Figure 5F; median 24.0 nm) than in acetone-treated skin (Figure 5E; median 3.2 nm). These results indicate that thinner collagen fibrils were separated by an enlarged interfibrillar space in DNFB-treated skin.

In skin samples stained with Cupromeronic Blue, fine filaments were observed around the collagen fibrils (Figures 4B, 4D, 4E and 4F). These filaments were DS, because they were mostly removed by chondroitinase ABC but not by chondroitinase

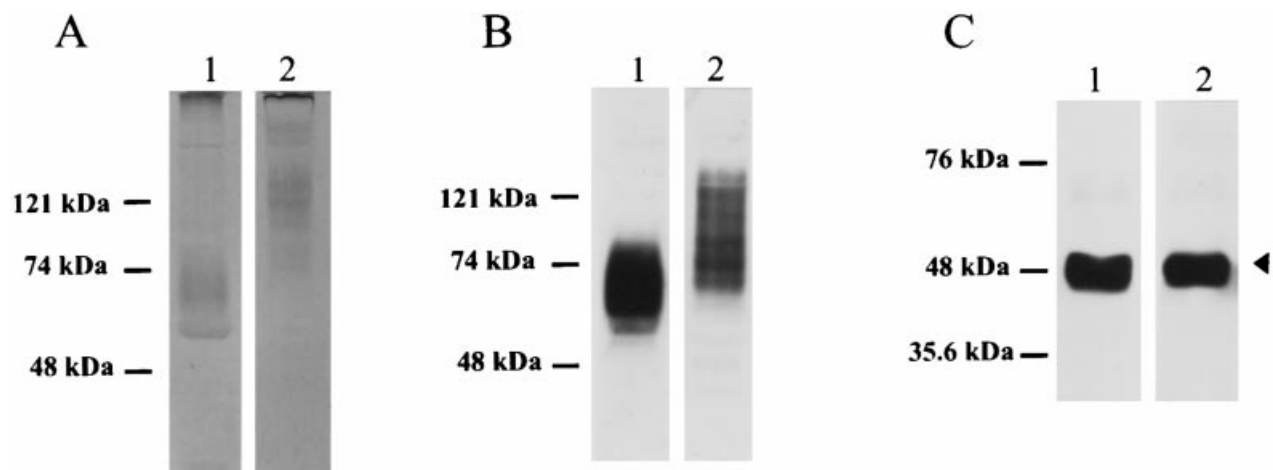


Figure 3 Analysis of decorin

SDS/PAGE of proteoglycan fraction [7.5% (w/v) gel] (**A**) and Western blotting of decorin (**B**) and DS-depleted core protein (**C**). Lanes 1, acetone-treated skin; lanes 2, DNFB-treated skin. The positions of molecular mass markers are indicated at the left.

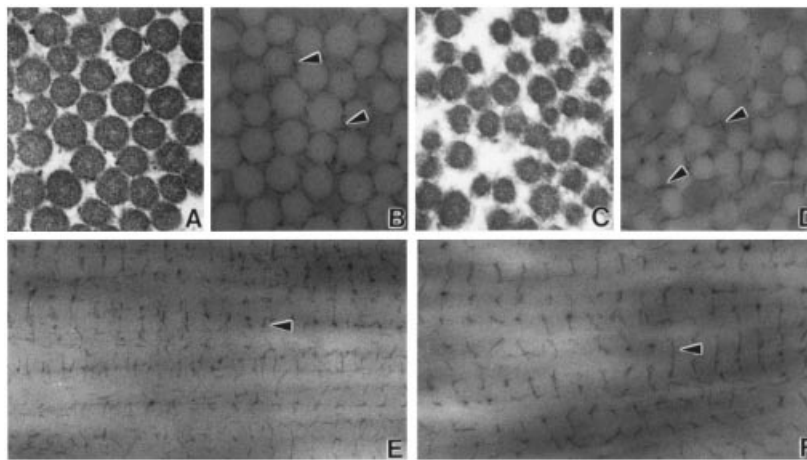


Figure 4 Electron photographs of collagen fibrils and DS filaments

Collagen fibrils (**A, C**) and DS filaments (**B, D, E, F**) of acetone-treated skin (**A, B, E**) or DNFB-treated skin (**C, D, F**) on day 15. Cross sections of collagen fibrils (**A–D**); sections longitudinal to collagen fibrils (**E, F**). Arrowheads in (**B, D, E, F**) indicate DS filaments. Scale bar, 100 nm.

ACII (results not shown). In longitudinal sections of collagen fibrils (Figures 4E and 4F), DS filaments approximately orthogonal to collagen fibrils were measured. The length was significantly longer in DNFB-treated skin (Figure 5H; median 50.8 nm) than in acetone-treated skin (Figure 5G; median 27.4 nm), although the distribution overlapped to some degree between the two groups. In cross sections, DS filaments in acetone-treated skin were located among tightly packed collagen fibrils that appeared paler than interfibrillar gaps with this staining (Figure 4B). Elongated DS filaments in DNFB-treated skin extended from the surface of collagen fibril across the enlarged interfibrillar space (Figure 4D). It is noteworthy that the histogram profiles of the surface-to-surface distance and the length of DS filaments in acetone-treated skin (Figures 5E and 5G) are quite different. The length of the DS filament was much

longer than the surface-to-surface distance in acetone-treated skin. This implies that most of the DS filaments did not run along the shortest distance between collagen fibrils, as observed in Figure 4(B). This was also true of DNFB-treated skin, as shown in Figures 4(D), 5(F) and 5(H).

DISCUSSION

Modulation of the molecular size of GAG has been shown several times. Bassols and Massagué [22] reported that transforming growth factor β (TGF- β) induced an increase in the molecular size of chondroitin sulphate/DS produced by cultured fibroblasts. An age-dependent decrease in the molecular size of chondroitin sulphate was found in the cartilage proteoglycan aggrecan [23]. Furthermore, the molecular size of DS isolated

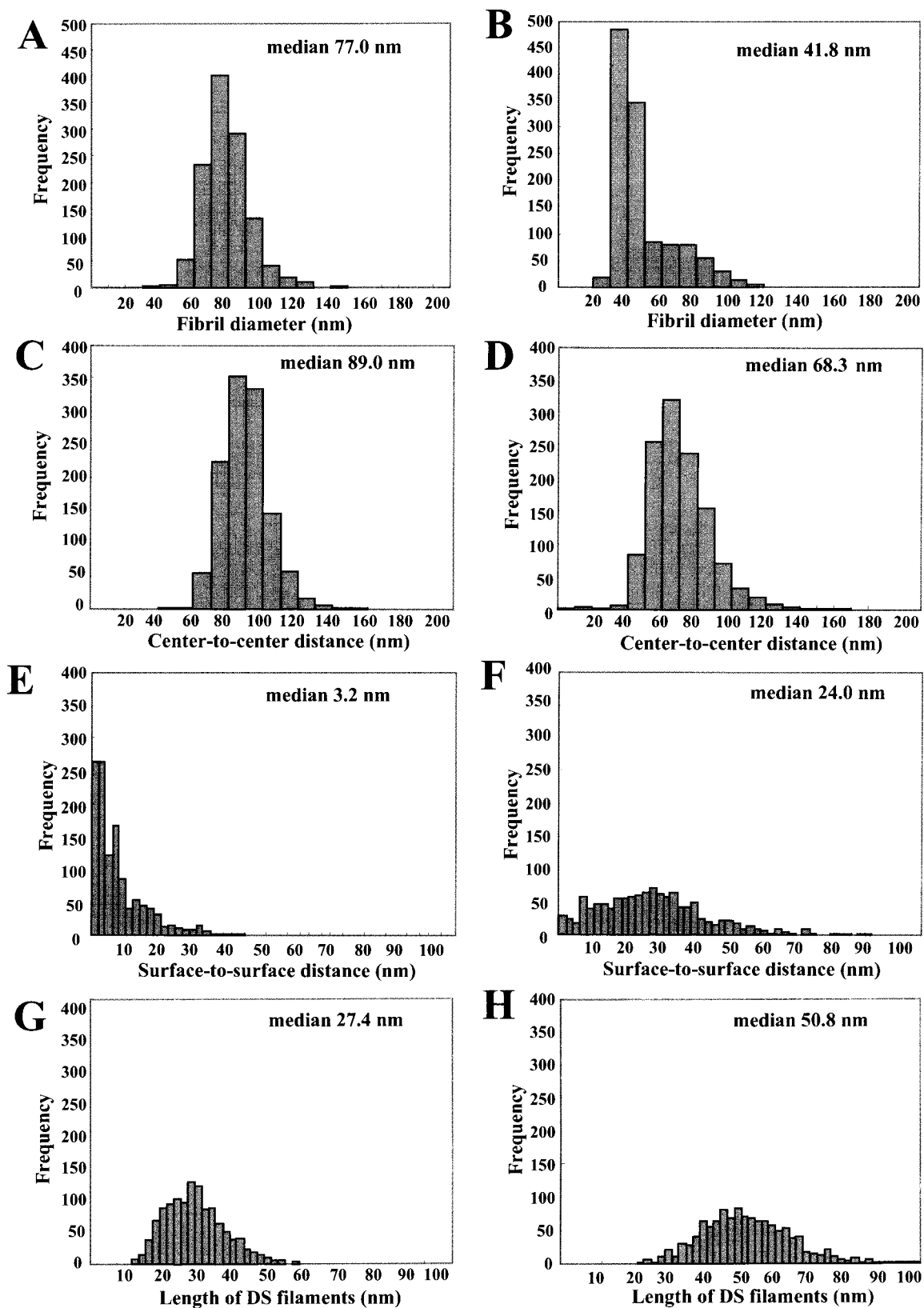


Figure 5 Histogram of collagen fibril size and DS length

Histogram of diameter of collagen fibrils (A, B), centre-to-centre distance (C, D), surface-to-surface distance (E, F) and length of DS filament (G, H) in acetone-treated skin (A, C, E, G) and DNFB-treated skin (B, D, F, H). Summary of three independent experiments; $n = 1200$.

from granulation tissue or tumour was larger than that from normal tissue [24]. These studies suggest that the length of GAG is controlled depending on physiological or pathological conditions. However, the biological significance of the altered GAG size, particularly that of DS, remains unclear.

In the present study, elongation of DS in healing skin was demonstrated not only by biochemical analyses but also by electron microscopy. On molecular sieve chromatography, whole DS isolated from skin was larger in DNFB-treated skin than in acetone-treated skin. The size of decorin DS was shown to be longer in DNFB-treated skin than in acetone-treated skin, although we do not exclude the possibility that the DS of biglycan is also elongated in healing skin. Furthermore, electron microscopic observation confirmed the elongation of the DS filament of decorin found on the surface of collagen fibrils in DNFB-treated skin.

A core protein of decorin binds non-covalently to the surface of collagen fibrils and suppresses the lateral growth of collagen fibrils [4–10]. In contrast, the decorin DS chain has been proposed to contribute to maintaining the spacing of collagen fibrils by forming an anti-parallel duplex and determining the distance between each collagen fibril [11]. This model is based partly on the fact that the fibrils are widely separated and DS is quite large (approx. 50 kDa) in the cornea, whereas in the sclera of the eye the collagen fibrils are close together and the DS is shorter (approx. 20 kDa) [25]. In the present study, ultrastructural morphometry revealed that collagen fibrils are packed tightly and uniformly in acetone-treated skin, but not in DNFB-treated healing skin. Healing of the skin was associated with a decrease in fibril diameter and centre-to-centre distance, but with an increase in the surface-to-surface distance between collagen fibrils. Thus shorter DS filaments in control skin were distributed among tightly packed collagen fibrils, whereas elongated DS filaments in healing skin were found among widely separated collagen fibrils. On the assumption that the mechanisms of DS regulating the distance of collagen fibrils in the cornea or sclera are also operating in the skin, our study suggests that the enlarged interfibrillar space in healing skin is controlled, at least in part, by the elongation of decorin DS.

Kischer and co-workers [26,27] showed that collagen fibrils in hypertrophic scar and granulation tissue were narrower and more widely spaced than those in normal skin. It was also reported that hypertrophic scar contains high amounts of versican and biglycan and smaller amounts of decorin in the early stages of scar formation [28], but that decorin increases in amount in later stages [29]. These studies suggest that the regulation of expression and distribution of proteoglycans is a critical step for tissue healing processes. In addition to the change discussed above, our present study demonstrates that the size of the GAG chains, particularly that of decorin DS, is controlled in healing processes, contributing to the arrangement of collagen fibrils in remodelling tissues.

It has been proposed that the length of GAG chains is determined by the relation between both the activities of β -N-acetylgalactosaminyltransferase and β -glucuronyltransferase and the number of core proteins available for polymerization processes [30,31]. It is tempting to speculate that such enzymes involved in DS polymerization are activated in the healing processes. In this connection, it is interesting to note that an increase in the molecular size of GAG has been reported in TGF- β -treated fibroblasts [22], healing wound and tumour [24], where TGF- β is probably important in remodelling the tissue. These studies suggest the possibility that TGF- β is involved in the elongation of DS in post-inflammatory healing. Further studies will be required to address the issue.

Taken together, the results of the present study suggest that the modulation of decorin DS in healing skin is important in tissue remodelling by organizing collagen fibrils separated by enlarged gaps.

We thank Dr T. Yamagata (The Japan Institute of Leather Research, Tokyo, Japan) for his critical reading of this manuscript.

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Received 22 November 2000/17 April 2001; accepted 1 June 2001