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Note

Sulfate composition of dermatan sulfate from scar tissue *

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Connective tissue injury triggers a series of cellular reactions that ultimately lead to wound repair. In the early stages of wound healing, cells produce and release macromolecules such as hyaluronic acid, a preponderant skin glycosaminoglycan (GAG) of the extracellular matrix[1,2]. In the late stages, however, sulfated GAGs are made in large concentrations, while hyaluronic production decreases. It is also interesting to note that cell differentiation and formation of collagen occur with the production of sulfated GAG[3–5].

The study of wound healing has concentrated on cell differentiation and migration as well as on the specific stages at which different tissue constituents are synthesized[5]. Little is known about the role of individual macromolecules such as proteoglycans on the mechanism of wound cicatrization. The dermatan sulfate (DS) proteoglycan should have specific function(s) during tissue restoration because of its possible involvement in controlling the growth and orientation of collagen fibers[6].

Because the healing of skin burns is accompanied by scar formation[7], and the content of small DS proteoglycan (Decorin) is known to be abnormally elevated in hypertrophic scar tissue[8], the role of this proteoglycan on the mechanism of wound repair cannot be ignored[9].

The present work shows analyses of DS isolated from hypertrophic and normal scars by Fourier-transform, proton nuclear magnetic resonance (FT-^{1}H NMR) and FT-infrared (IR) spectroscopic methods.

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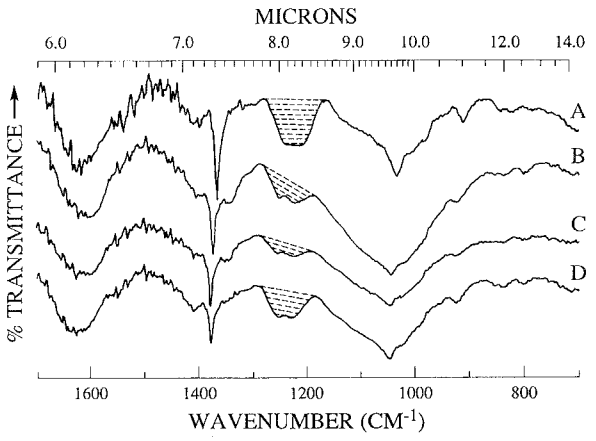

Fig. 1. Infrared spectra of standard and scar dermatan sulfate: A, human skin DS standard; B, normal human skin DS used as control; C, normal scar DS; D, hypertrophic scar DS. Pellets were prepared with 100 mg KBr, and the amounts of GAG are indicated in Table IV. The dashed areas were used to calculate sulfate as described elsewhere[13].

Scar and normal skin GAG obtained upon chemical removal of the core proteins from the DS proteoglycans previously reported[10] had the same mobility on cellulose polyacetate membranes during one-dimensional electrophoresis. Such mobility was, however, a bit faster than that of DS standard (not shown). These GAGs were also degraded by chondroitinase ABC but not by chondroitinase AC II or *Streptomyces* hyaluronidase[10,11]. Their IR and FT-^{1}H NMR spectra were characteristic of DS[12] (Figs. 1 and 2). These data demonstrated these GAGs to be DSs.

The DSs under discussion had the following chemical characteristics: 30–35% IdoA, with GlcA being the major uronic acid (Table I); around 100% (mol/mol) *N*-acetylation of hexosamine (Table II); and similar values for uronic acid : hexosamine ratios (Table III).

Sulfate was the DS component altered significantly in normal and hypertrophic scars. As shown in Table IV, normal scar DS displayed a 44% (w/w) drop in sulfate, while hypertrophic scar DS had a 22% (w/w) sulfate increase compared to the control. It is worth noting that the sulfate values of scar and control DSs which originated from abdominal tissue were lower than those estimated by the same method for standard DS of breast skin[13]. Such differences suggest variation in the sulfate composition of DS with skin site.

Scar DSs had characteristic sulfate contents which differed from that of the control, though all patients were in the 5–20 year old range, and the skin was abdominal. These data, therefore, suggest a direct relationship between hypertrophy of scar tissue and DS oversulfation.

Because the values for the parameters of DS standard analyzed with scar DSs turned out to be the same as those previously determined[12,13], the data reported here for these DSs are not the results of experimental variations.

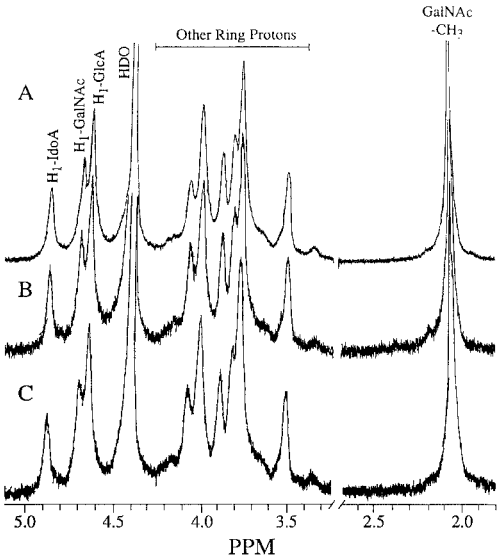

Fig. 2. Proton NMR spectra of scar dermatan sulfate: A, DS of human skin was used as the control; B, normal scar DS; C, hypertrophic scar DS. Spectra, which appeared as those of human skin DS standard[13], were recorded at 60°C using a Varian VXR-500 MHz spectrometer, with 64 scans accumulated in 15 min. Residual H in D_2O set at 4.41 ppm was the external reference[12].

EXPERIMENTAL

Materials.—Hypertrophic abdominal scar tissue from burn wounds was obtained by surgical scar removal from 12 male and female patients, 5–16 years of age at the Shriners Burns Institute, Boston Unit. The normal scar tissue also from abdominal burn wounds was excised by surgery on 12 male and female patients 8–20 years old. Scar type was established by histochemical tests conducted as indicated elsewhere[14]. The average ages of the hypertrophic and normal scar tissues,

TABLE I

Uronic acid content of standard and scar dermatan sulfate

Dermatan sulfate	Chemical shifts of H-1 [a]			% L-IdoA [c] (mol/mol)
	L-IdoA (4.94 ppm)	D-GlcA (4.68 ppm)	Total [b]	
DS standard	1.15	1.50	2.65	43.0
Control	3.90	9.20	13.10	29.8
Normal scar	3.10	7.40	10.50	29.5
Hypertrophic scar	2.80	5.10	7.90	35.0

[a] Expressed as integration units. [b] Sum of integration values at 4.94 and 4.68 ppm which represent total uronic acid[12]. [c] Percentage L-IdoA relative to total uronic acid. The integration values of H-1 of L-IdoA and D-GlcA shown in Fig. 2 were employed to calculate the relative concentrations of these uronic acids. All calculations were done in triplicate, and the average is reported. See text for experimental conditions.

TABLE II

Percentage *N*-acetylation of D-galactosamine in standard and scar dermatan sulfate

Dermatan sulfate	Chemical shifts of D-GalNAc [a]		% *N*-Ac [b] (mol/mol)
	H-1×3 (4.74 ppm)	$-CH_3$ (2.10 ppm)	
DS standard	5.40	3.30	61
Control	17.10	17.10	100
Normal scar	12.90	12.90	100
Hypertrophic scar	10.80	10.60	98

[a] Expressed in integration units. [b] Percentage hexosamine *N*-acetylation[12] calculated by using the spectrum shown in Fig. 2 and the following formula:

$$\frac{\text{Integration value at 2.10 ppm}}{3\times(\text{Integration value at 4.74 ppm})}\times 100$$

Each point is the average of three different determinations.

TABLE III

Uronic acid:hexosamine ratios of standard and scar dermatan sulfate

Dermatan sulfate	Uronic acid [a]	GalNAc [b]	Uronic acid:hexosamine
DS standard	2.65	1.80	1.5:1
Control	13.10	5.70	2.3:1
Normal scar	10.50	4.30	2.4:1
Hypertrophic scar	7.90	3.60	2.2:1

[a] Sum of integration values at 4.94 (H-1 of L-IdoA) and 4.68 ppm (H-1 of D-GlcA)[12]. [b] Integration values at 4.74 ppm (H-1 of hexosamine) in integration units[12].

TABLE IV

Sulfate composition of standard and scar dermatan sulfate determined by infrared spectroscopy

Dermatan sulfate	DS/KBr [a] (μg)	IR band [b] (mg)	SD (×10⁴)	SO_4^{2-} [c] (μg)	SO_4^{2-} [d] (%)	Change [e] (%)
DS standard [f]	390	22.1	5.0	35.0	8.9	
Control	330	12.4	6.3	19.5	5.9	
Normal scar	350	7.6	3.3	9.1	2.6	44↓
Hypertrophic scar	370	17.2	4.1	26.6	7.2	22↑

[a] Micrograms DS in 100 mg KBr pellet prepared as indicated elsewhere[13]. [b] Weight of S=O asymmetric stretching band, dashed area on IR spectra shown in Fig. 1. SD standard deviation ×10⁴. [c] Sulfate in total sample determined on a standard curve[13]. [d] Expressed in μg SO_4^{2-}/100 μg DS. [e] Change relative to the control: ↓, decrease; ↑, increase. [f] DS of human breast skin also analyzed previously (Table 1 in ref. 13). Band areas around 1230 cm^{-1} (dashed regions, Fig. 1) which originated from asymmetric stretching of S=O bonds in sulfate groups were traced on IR transparency film cut and weighed. Each value represents an average of 3 determinations conducted in triplicate.

following injury, were 55 months and 71 months, respectively[10]. For the control, full thickness, human skin freshly removed by abdominal reduction of 10 male and female patients aged 6–16 years was used. The latter reductions were performed at

the Massachusetts General Hospital. Only normal tissue tested histologically was employed[14]. Skin from each group was pooled separately *.

Purification and characterization of the DS proteoglycan.—GAGs were obtained by removal of the protein cores from the DS proteoglycans reported previously[10]. Briefly, skin powder from normal and post-burn scar skin was stirred in 4 M guanidine · HCl–0.05 M NaOAc–0.05 M Tris, pH 7.6. This solution contained the proteolytic enzymes described elsewhere[10]. The DS proteoglycan was isolated from this extract by chromatography on EAE-cellulose, followed by differential EtOH precipitations[10,11].

The identity and purity of the proteoglycans were established by a variety of methods including digestion with the lyase enzymes, chondroitinases AC II (EC 4.2.2.5) and ABC (EC 4.2.2.4), in addition to *Streptomyces* hyaluronidase (EC 4.2.2.1) and chemical analyses of the corresponding products[10,11].

To remove the proteins, proteoglycans were treated with 0.05 M NaOH–1 M $NaBH_4$ at 37°C for 72 h. The GAGs were purified on a DE-52 column as described elsewhere[7].

Purification of DS for Fourier-transform, proton nuclear magnetic resonance spectroscopic studies.—Dermatan sulfate (1 mg) obtained as indicated above was dissolved in 0.5 M NaCl, pH 5.09, adjusted with HCl. Absolute EtOH (2.5 mL) was added, and the resulting mixture was stored at 4°C overnight. Because no precipitate formed, additional NaCl solution (200 μL) was added, and the solution was again stored at 4°C overnight. The white precipitate obtained was removed by centrifugation at 3000*g* and 4°C for 15 min, washed 3 times with 80% (v/v) aqueous EtOH, lyophilized, and stored over P_2O_5. The control had all reagents but DS and displayed no precipitate upon addition of EtOH.

Purified DS (330–400 μg) was dissolved in 0.3 mL D_2O (99.996 atom%) (Aldrich Chem. Co. Inc., Milwaukee, WI 53233, USA), lyophilized, redissolved in the same D_2O and relyophilized 3 times. The dry sample was dissolved in the same D_2O (0.4 mL) under a dry N_2 atmosphere and used for the NMR investigations.

Preparation of DS for infrared spectroscopy.—DS (330–400 μg) precipitated with EtOH as described above was dissolved in highly pure water (1.0 mL) and combined with KBr (100 mg). The homogeneous solution obtained was lyophilized. A dry KBr–GAG mixture was employed for preparing pellets that were used immediately for IR spectroscopic analyses.

* Both Shriners Burns Institute and Massachusetts General Hospital have a standard form for the patients to sign when getting admitted. In this form patients authorize the hospital to retain any organs or tissues removed surgically, use them for scientific or educational purposes or dispose of them in accordance with established practice. All tissue employed in this study was post-surgical, and disposable, and the only data available to the researchers was age and sex. Therefore, the study was exempt from IRB approval under exemption No. 5.

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