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Glycosaminoglycan Alteration in the Skin of Children with Classical Ehlers-Danlos Syndrome

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Three children with classical Ehlers-Danlos syndrome were studied. Electron microscopic observation of the skin demonstrated a hyaluronic acid-dominant glycosaminoglycan structure in the intercellular matrix. Biochemical analysis showed a significant increase in hyaluronic acid content, as compared with the skin of sex- and age-matched children, including a non-affected brother of a patient. In addition, enhanced hyaluronic acid synthesis was demonstrated in vitro, using a patient's dermal fibroblasts. Analysis of the collagen showed no apparent abnormalities. *Key words: Skin glycosaminoglycan.* (Received April 3, 1985.)

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Ehlers-Danlos syndrome (EDS) is a hereditary disorder characterized by hyperelastic skin, easy bleeding and hypermotility of joints. EDS, which was initially classified into 7 variants (1), was understood to be a collagen synthesis-disorder, according to the results of biochemical research (2, 3 and others). However, the pathomechanism of classical EDS, such as Types I, II and III, remains to be elucidated. Shinkai et al. (4) discussed an abnormality in the structural glycoprotein as well as a procollagen peptidase deficiency in a patient with EDS Type I. In addition, in other subsets, a patient with Type V having normal lysyl oxidase activity (5) and one with Type VI showing normal hydroxylysine content with no deficiency in lysyl hydroxylase (6) have been described. Furthermore, new variants, showing alterations other than in collagen have been found (7–9). In the present study, we have analyzed connective tissue changes in children with classical EDS.

MATERIAL AND METHOD

Three patients with EDS (5, 10 and 5 yrs: cases 1, 2 and 3 in mentioned order; all males) were examined. Clinical features of these patients are shown in Table 1. They had findings compatible with those of Type I (gravis) or II (mitis). Hyperelastic skin was excised from the lower back (case 1) or forearm (cases 2 and 3) for morphological and biochemical study. As a control, the uninvolved skin from 3 age and sex-matched individuals (1 specimen each from the upper arm (6-yr-old boy with von Recklinghausen's disease), forearm (10-yr-old boy with psoriasis vulgaris), and back (non-affected brother of case 3, 7-yr-old) similarly studied.

Histological examination. The skin specimen was fixed with formo-ethanol (1 : 9) solution containing 1% cetyltrimethylammonium bromide (CTAB), dehydrated and embedded in paraffin. Sections were stained with hematoxylin-eosin, Weigert staining for elastic fibers, and colloidal iron-PAS. In addition, the skin proteoglycans were immunohistochemically examined after the procedure described in a previous report (10): Six μm thick cryostat section was stained by indirect immunofluorescent technique, using rabbit antiserum against bovine cartilage proteoglycan diluted 1 : 80 and FITC-labeled swine anti-rabbit immunoglobulin (Dakopatts) diluted 1 : 40. (Hyaluronic acid structure is not stained by this method.)

Electron microscopic study. Ultrastructural demonstration of skin glycosaminoglycan (GAG) was carried out according to the method described (11), along with routine electron microscopic methods.

Routine ultrathin sections were double-stained with uranyl acetate-lead, and observed with the JEM-100C electron microscope. The GAG structure was observed without any contrast-staining.

Biochemical examinations

Analysis of skin. Collagen content expressed as hydroxyproline per defatted dry weight was measured by the method of Woessner (12), after hydrolysis of the dermis with 6 N HCl at 130°C for 3 h. Collagen types were examined by a modified procedure of Weber (13). The homogenized dermis was treated with CNBr in 70% formic acid at 40°C for 4 h, lyophilized, and applied to 15% SDS-polyacrylamide gel electrophoresis. CNBr-peptides were stained with 0.025% Coomassie brilliant blue. The stainability of CB8 of α_1 (I) and α (III) was determined by a densitometer, and the ratio of Type I/Type I+III was calculated, based upon the molecular weights-estimation by Seyer et al. (14):

$$\frac{\text{Type I}}{\text{Type I+III}} = \frac{\alpha_1\text{(I) CB8/2} \times \frac{285\,000}{24\,000}}{\alpha_1\text{(I) CB8/2} \times \frac{285\,000}{24\,000} + \alpha\text{ (III) CB8/3} \times \frac{280\,000}{12\,000}} \times 100\ (\%)$$

Skin GAG was isolated by a modified procedure of Schiller et al. (15). The dermis was minced, defatted with acetone, dried under vacuum and weighed. Then the sample was homogenized in 2% NaOH and allowed to stand overnight with constant stirring. After dialysis against running water for 24 h, the sample was digested with 1.5% (W/W) Pronase E (Kaken Kagaku, Tokyo) in 100 volumes (V/W) of 0.1 M Tris-HCl buffer (pH 7.8), containing 2.5 mM $CaCl_2$ at 50°C for 24 h. After readjusting the pH to 7.8, the digestion was repeated with the addition of Pronase E. The sample was deproteinized with trichloroacetic acid (TCA) (10%, final). After centrifugation, the crude GAG of the supernatant was precipitated with CTAB, after the procedure of DiFerrante Rich (16). Uronic acid of the GAG was determined by the method of Blumenkrantz & Asboe-Hansen (17). The distribution pattern of constituent GAGs was calculated by comparing, with a densitometer, the stainability of spots developed on the cellulose acetate membrane with that of standard GAGs (18).

Synthesis of collagen and GAG by dermal fibroblasts. (^{3}H) proline-labeled procollagen synthesis by dermal fibroblasts in monolayer culture was examined by the procedure of Tan et al. (19). Dermal fibroblasts were cultured from explants of biopsied skin, and 3rd passage cells were used for this study. For ^{35}S-GAG synthesis, dermal fibroblasts (3rd passage), adjusted to a concentration of 1×10^5/ml, were incubated in a plastic dish (9.6 cm ⌀) containing Eagle's minimum essential medium (MEM) with 10% dialyzed, fetal calf serum (FCS) and 100 μM/ml sodium sulfate at 37°C, under atmosphere of 5% CO_2. To confluent cells, 30 μCi/ml (^{35}S) H_2SO_4 (New England Nuclear) was added, and cells were incubated for a further 24 h. NaOH (2%, final) was added to the medium. After being allowed to stand overnight, the sample was dialyzed against water, and deproteinized with TCA, using a similar procedure to the isolation of the skin GAG. After centrifugation, the supernatant was dialyzed and lyophilized as ^{35}S-GAG sample. The radioactivity of part of the sample was counted with a liquid scintillation spectrometer. The rest of the sample was used for the determination of the distribution pattern of synthetized sulfated GAGs, by the method of Saito et al. (20). (^{3}H) glucosamine-labeled GAG synthesis was examined after a modified procedure of Goldberg & Toole (21):

Table I. *Clinical features of children with Ehlers-Danlos syndrome*

Age	Sex	Onset	Hyperelasticity of the skin	Bruising	Hyperextensibility of joints	Complication	Heredity
5	M	At birth	+ With LIN's sign	−	+	−	Father affected
10	M	At birth	+	−	+ With subluxation of femoral joint	Decreased DLco in lung test	Not identified
5	M	Since infancy	+	+	+	Hyperelastic nasal and auricular cartilage	Not identified

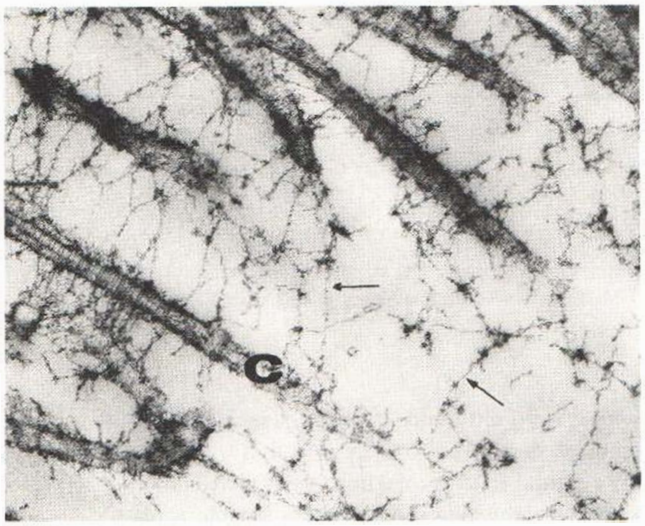

Fig. 1. Electron micrograph showing the hyaluronic acid (arrow) without attachment of proteoglycans, observed in the intercellular matrix of skin from classical Ehlers-Danlos syndrome. Collagen fibrils (C). Fixed with CTAB, and post-fixed with 0.1 M phosphate buffer (pH 7.4) containing 0.05% ruthenium red and 2% OsO_4. ×65 760.

Cells (3rd passage) adjusted to 1×10^5/ml were incubated in a plastic dish (9.6 cm ϕ) containing Dulbecco's MEM with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin until confluent, at 37°C under an atmosphere of 5% CO_2. Then, 10 μCi/ml (^{3}H) glucosamine (Amersham) was added to the medium and incubated further for 24 h. The medium examined was digested with 1 mg/ml Pronase E in 100 volumes (V/V) of 0.1 M Tris-HCl buffer (pH 7.8) containing 2.5 mM $CaCl_2$ at 50°C for 24 h. The pronase was inactivated by boiling for 15 min. Crude GAG was precipitated by 3 volumes of ethanol with 1.3% potassium acetate. The precipitate, dissolved in 1.3% potassium acetate, was divided into 2 parts (2 ml). To one part, 10 units of streptomyces hyaluronidase (Amano) was added, and it, as well as the other sample without enzyme, was incubated for 24 h at 37°C. Then CTAB (1%, final) was added to it and allowed to stand at 4°C overnight. After centrifugation, the pellet was washed twice with 0.05% CTAB containing 0.05 M NaCl, then the radioactivity was determined in a liquid scintillation counter. Hyaluronic acid synthesis was expressed as the difference between the radioactivities of both samples.

RESULTS

Histology

In all of the skin specimens from EDS, the dermis showed a moderate interstitial edema. Paralleling the interstitial edema, colloidal-iron-positive material was increased between PAS-positive collagen fibers. However, immunofluorescent staining of the skin proteoglycans showed no evident changes, as compared to the control skin, though an intense fluorescence was observed in the subepidermal zone and perivascular area in both EDS and control. Elastic fibers exhibited no remarkable changes.

Electron microscopic observation

Collagen fibrils showed no significant alterations. Neither abnormal diameter of collagen fibrils nor loose bundle-formation of collagen fibrils was seen. In contrast, the intercellular matrix, stained with Ruthenium red, was enriched in fine networks of long, straight filaments of about 3.0–7.0 nm in thickness with no attachment of short, irregularly curled filaments (proteoglycan chains), demonstrating the presence of only a hyaluronic acid structure (11), particularly in areas around loosely arranged collagen fibrils (Fig. 1). Although the control skin demonstrated the GAG structure of normal skin (proteoglycan chains attaching to the hyaluronic acid filaments), such a structure occurred in fewer areas in the skin of EDS.

Table II. *Collagen changes in boys with classical Ehlers-Danlos syndrome*

Sample	Examination	EDS (n=3)	Control (n=3)
Skin	Hydroxyproline (μg/mg)	80.1±2.9	77.5±4.7
	Type I/Type I+III (%)	72.2±2.9	71.9±8.2
Fibroblast	(^{3}H) procollagen (dpm/dish×10^{-6})	1.5±0.2	1.4 (n=1)[a]
	Type I/Type I+III of (^{3}H) procollagen (%)	64.7±4.7	64.8 (n=1)[a]

Values of 3 samples are expressed as mean ±SEM. [a] Non-affected brother of case 3 with EDS.

Biochemical analysis

The results are summarized in Table II. There was no difference in skin collagen content and molecular species as compared with control specimens. Synthetic activity and the molecular species of procollagen produced by cultured EDS dermal fibroblasts also did not differ from those of control fibroblasts. In contrast, the skin GAG content (uronic acid) showed a high value in specimens from cases 1 and 2 (4.1 and 2.0 μg/mg). In particular, hyaluronic acid was significantly increased, as compared with that of the control skin. In addition, fibroblasts from EDS demonstrated a higher value in both ^{3}H-hyaluronic acid and ^{35}S-dermatan sulfate synthesis, in particular, in hyaluronic acid synthesis, as compared to control fibroblasts.

DISCUSSION

The present study revealed an increase in skin hyaluronic acid in children with classical EDS. Paralleling this result, hyaluronic acid synthesis by dermal fibroblasts from a patient with EDS was also incresed. Electron microscopic study revealed the presence of hyalur-

Table III. *Glycosaminoglycan changes in boys with classical Ehlers-Danlos syndrome*

HA=hyaluronic acid; DS=dermatan sulfate; C4S=chondroitin 4-sulfate; C6S=chondoritin 6-sulfate; CHase=chondroitinase ABC and AC

Sample	Examination	EDS (n=3)	Age, sex-matched control (n=3)
Skin	Total GAG as uronic acid (μg/mg)	2.4±0.9	1.0±0.2
	Hyaluronic acid as uronic acid (μg/mg)	1.9±0.6*	0.6±0.2
Fibroblast	(^{3}H)glucosamine-labeled GAG synthesis (dpm/dish×10^{-4})		
	HA	140 (n=1)[1]	8 (n=1)[2]
	non-HA	35	9
	^{35}-S-GAG synthesis (dpm/dish×10^{-4})	3.4±0.5	1.2 (n=1)[2]
	distribution pattern (%)		
	DS	33.9±2.4	18.0
	C4S	18.8±4.4	18.3
	C6S	17.2±2.1	39.8
	CHase-resistant	30.1±5.1	23.9

Values in 3 samples are expressed as mean±SEM. [a] Case 3 with EDS. [b] Non-affected brother of case 3.
* Significant at Student's t-test vs. control (p<0.01).

onic acid structures alone in many areas of the skin specimen, and histological results agreed with it. Our previous investigation (11) confirmed that the matrix GAG of the normal skin is composed of hyaluronic acid filaments attached to proteoglycan chains, that is, a proteoglycan aggregate-like structure. However, in pretibial myxedema or lichen myxedematosus, in which the hyaluronic acid content is extremely increased, a hyaluronic acid structure alone was observed, along with marked decrease in the number of collagen fibrils. In contrast, the sclerotic skin of systemic sclerosis was characterized by the random proteoglycan deposition between collagen fibers (10). These findings suggest that an appropriate ratio of hyaluronic acid to proteoglycans, is necessary in normal collagen fibrils formation. Although an apparent alteration in collagen fibrils was not observed in specimens examined, Vogel et al. (22) noted an increase in thick collagen fibrils, suggesting a disturbance in collagen aggregation in classical EDS. Therefore, the increse in skin hyaluronic acid demonstrated in the present study, could be related to the pathogenesis of skin changes characteristic of classical EDS.

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