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CROSS-LINKING OF COLLAGEN IN THE X-LINKED EHLERS-DANLOS TYPE V

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SUMMARY

The activity and antigenicity of the collagen crosslinking enzyme, lysyl oxidase, and the proportions of reducible crosslink in skin biopsies from Ehlers-Danlos Type V subjects were equivalent to those of control skin. These results reveal that both the potential for crosslinking, and the ability to form reducible crosslinks is present in Ehlers-Danlos syndrome Type V subjects, clearly demonstrating that the defect in this disorder is not due to a defective crosslinking mechanism.

The Ehlers-Danlos syndrome has been classified by clinical and biochemical criteria into seven different sub-groups (1, 2). The molecular defect in the dominantly inherited Types I to III has not been identified but the disorders in the recessively inherited Types IV to VII have recently been characterised. The dermis of Ehlers-Danlos syndrome Type IV subjects was found to be deficient in Type III collagen (3) whilst Type V VI and VII were found to have dramatically reduced activity of lysyl oxidase (4), lysyl hydroxylase (5) and procollagen peptidase (6) respectively. During a study of Types I, II and V we could find none of these previously identified defects. In view of the report by Di Ferrante et al (4) that the Type V Ehlers-Danlos syndrome was due to deficiency in the lysyl oxidase activity we have re-examined the Type V subject in more detail. In this paper we report that the lysyl oxidase activity and cross-linking of the sex-linked Ehlers-Danlos syndrome Type V are comparable with normal skin despite the clinical defects.

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MATERIALS AND METHODS

Clinical details

J.W., a 33 year old male, was identified as having the classical features of Ehlers-Danlos syndrome Type V by Beighton (7). He has mild joint mobility, marked skin extensibility, a tendency to easy bruising, molluscoid tumours, and has had recurrent dislocation of his shoulders. He has a brother who is similarly affected, but no other positive family history (Table 1). Two other brothers, D.S. and A.S., were also identified as Ehlers-Danlos Type V subjects (their clinical features are also described - Table 1).

Preparation of intact collagen fibres

The skin biopsies were cleaned of adhering fat and muscular tissue, shredded with a scalpel, and washed with copious amounts of physiological saline, 0.9% NaCl pH 7.4, to remove soluble proteins and glycosaminoglycans.

Reduction of the intact collagen fibres

The samples of intact collagen fibres from skin biopsies of J.W., B.W., D.S. and A.S. were suspended in 0.9% NaCl pH 7.4 and reduced with tritiated

TABLE 1 CLINICAL FEATURES OF EHLERS-DANLOS SYNDROME SUBJECTS

Patient	Joint Mobility	Skin Extensibility	Scarring	Bruising	Molluscoid Pseudotumours	Other Features
Family 1						
J.W. (33 years)	Yes (mild)	Yes (marked)	No	Yes	Yes (moderate)	Recurrent dislocation of shoulders
B.W. (48 years)	Yes (mild)	Yes (marked)	Yes (mild)	Yes (Marked)	Yes	Haematoma
Family 2						
D.S. (22 years)	Yes (mild)	Yes	Yes	Yes	Yes	Epicanthus
A.S. (30 years)	Yes (mild)	Yes	Yes	Yes	No	Pes planus

J.W. is the patient under current study.

Grandparents - unaffected

John has two children - 1 son unaffected
1 daughter unaffected

Brian has two children - 1 son unaffected
1 daughter unaffected

potassium borohydride (KB^3H_4) diluted to 10 m Ci/mmol with non-radioactive (KBH_4) (8). For direct comparison with age matched controls, the KB^3H_4 was dissolved in saline (0°C) and equal portions of the solution used to reduce the suspended fibres. The reaction was allowed to proceed for one hour, after which time acetic acid was added to a final pH 4, the suspension dialysed against distilled water overnight, then freeze dried and weighed.

Identification of reducible crosslinks

The reduced samples were hydrolyzed in boiling 6N HCl for 24 hours, and the hydrolysate analysed by ion-exchange chromotography using the Technicon analyser with pyridine-formate buffers as described previously (8). The reducible components were identified in the collected effluent by determining the tritium activity of an aliquot from each fraction in the Packard Scintillation Counter using Brays solution (9).

Confirmation of the identity of the radioactive components was achieved by analysis against authentic samples on the long basic column (60 cms) of the Beckman 120 C amino acid analyser.

SDS acrylamide gel eletrophoresis

The skin samples were partially solubilized by heating in 2% SDS + 2% mercaptoethanol for two hours at 40°. The insoluble material was removed by centrifugation and the soluble material analysed by SDS polyacrylamide gel electrophoresis in borate buffer as previously described in detail (10).

Determination of lysyl oxidase activity

The weighed skin biopsies from J.W. and control were homogenised with a tissue grinder in cold 6 M urea, 0.05 M tris pH 7.5 (1 cc per mg tissue). Following centrifugation at 17,000 x g for 10 minutes, the pellet was again homogenised in the same buffer. The supernatants were dialysed against 0.15 M NaCl, 0.1 M $NaHPO_4$, pH 7.8.

Lysyl oxidase activity was measured by tritium release from labelled chick calvaria collagen substrate (11). Aliquots of skin extracts from both propositus and control subjects, given in Table 2, were incubated for two hours at 37°.

TABLE 2 DETERMINATION OF LYSYL OXIDASE ACTIVITY BY RELEASE OF TRITIUM

	Net ^3H Release *	Net ^3H Release/mg skin extracted
Ehlers-Danlos Syndrome V 33 years old	164	67.8
Control 40 years old	96	26.4

* Each extract was incubated with 844,000 cpm of lysine 6^3H labelled chick calvaria substrate for two hours as previously described (18)

Double immunodiffusion

Antiserum directed against purified human skin lysyl oxidase was produced in an adult rabbit. The presence of lysyl oxidase antigen in control and Ehlers-Danlos syndrome V skin samples was assayed with a monospecific rabbit anti-human skin lysyl oxidase antisera (Fu and Siegel in preparation) by Ouchterlony double diffusion (12). Precipitation bands were photographed after incubation for 48 hours at 4°C.

RESULTS

Reducible crosslinks

The reduced crosslinks hydroxy-lysinonorleucine (OH-LNL) and histidinohydroxymerodesmosine (HHMD) were found to be present in the dermal collagen from both the normal and all four of the Ehlers-Danlos syndrome Type V subjects. No significant difference could be detected between the crosslink patterns of the Ehlers-Danlos Syndrome subjects and the age matched controls. The high proportion of hexosyl lysines is typical of the normal pattern for mature dermal collagen (Fig. 1a).

Thermally stable crosslinks

Comparison of the solubilised collagen components on SDS acrylamide gel electrophoresis of the Ehlers-Danlos Type V and control skins revealed no significant difference. In both cases high molecular weight chains were present, clearly demonstrating the presence of stable crosslinks.

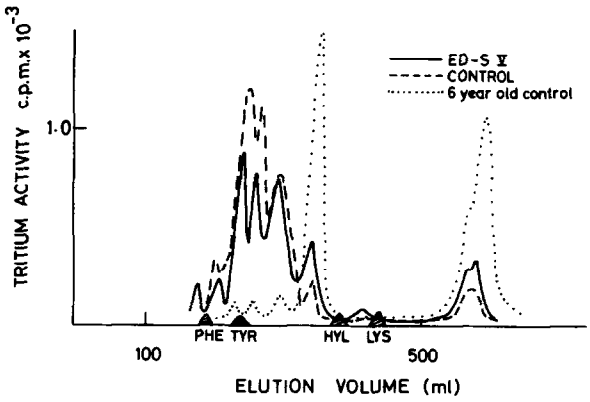

Fig. 1(a): Electron chromatogram of radioactive components separated from an acid hydrolysate of Na B^3H_4 reduced dermal collagen ——— Ehlers-Danlos Syndrome V subject. - - - - control age 33; ····· control age 6 yrs.

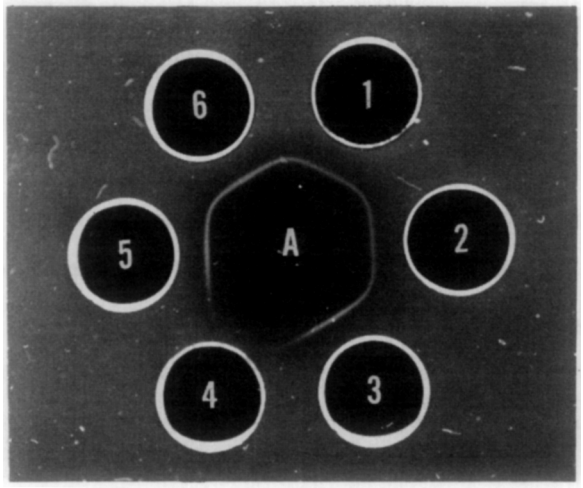

Fig. 1 (b): Ouchterlony double diffusion pattern. A is the antiserum against normal human skin lysyl oxidase; wells 1 and 5, extract from control skin; wells 2, 3 and 6 extract from Ehlers-Danlos Syndrome V skin; well 4 boiled extract from Ehlers-Danlos Syndrome V skin.

Lysyl oxidase activity

The lysyl oxidase activity in the skin of the propositus (J.W.) was more than twice the control (Table 2.). The Ouchterlony technique shows that protein(s) with antigenic activity corresponding to lysyl oxidase is present in the skin extracts of the patient with Ehlers Danlos Type V (Fig. 1b). The antigenicity appears identical to that in the control skin extract.

DISCUSSION

The activity and antigenicity of the crosslinking enzyme, lysyl oxidase, and the proportion of reducible crosslinks present in skin biopsies from this and other Ehlers-Danlos Type V subjects were not reduced in comparison to the skin of control subjects. In addition, SDS polyacrylamide gel electrophoresis of the soluble extract from the skin revealed the presence of high molecular weight components, confirming the presence of thermally stable crosslinking. Thus both the potential to form crosslinks and the presence of actual crosslinks has been clearly demonstrated. The similarity

of the crosslink profiles, e.g. the presence of hexosyl lysines (13) indicate that the reducible crosslinks undergo the same maturation process as normal skin (8) and secondly that no new reducible components are present.

These findings contrast with the study of an Ehlers-Danlos syndrome Type V subject by Di Ferrante et al (4) who reported a marked deficiency in the lysyl oxidase activity and concluded that the consequent lack of crosslinks would account for the clinical features. In that study, lysyl oxidase activity in lyophilized fibroblast tissue culture media after reconstruction in assay buffers was measured. The reported results may have been due to the known poor solubility of lysyl oxidase in buffers lacking urea (14). Alternatively, low activity also occurs when cells are not proliferating rapidly (15). Direct measurement of the activity from affected tissue as done here should minimise these and other potential artefacts.

The absence of crosslinks would lead to continuous extension of the fibres as they slipped past each other under tension until rupture occurred. In this situation there would be no restoring force, whereas in the Ehlers-Danlos syndrome subjects the skin returns to its normal position after hyperextension. This is reflected in the stress-strain analyses carried out on Ehlers Danlos sybdrome skin in which the large extensive region is followed by a region in which the stress-strain relationship is linear and parallel to the control tissue (16), that is, the fibrils must be crosslinked and virtually inextensible at this stage. The long extensible region is therefore not due to the absence of crosslinks within the fibril, but the packing of these fibrils into tight bundles. This suggestion is supported by the electron microscope studies; the transmission electron microscope showing fibrils of a normal structure, where, at a high morphological level, the scanning electron microscope reveals that the fibre bundles are less tightly packed (17).

In summary, we have clearly shown that the disorder is not due to a defective crosslinking mechanism, and suggest that the defect lies in the aggregation of the fibres into bundles.

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