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Patients with Ehlers–Danlos Syndrome Type IV Lack Type III Collagen

(connective tissue/fibroblasts/heritable diseases)

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ABSTRACT **One of the genetically distinct collagens (type III) normally found in skin, aorta, and intestine is missing from the tissues of patients with the Ehlers–Danlos syndrome type IV. While skin fibroblasts from other individuals synthesize both types I and III collagen, Ehlers–Danlos syndrome IV cells synthesize only type I. These results suggest that the fragile skin, blood vessels, and intestines of Ehlers–Danlos syndrome IV patients result from an absence of type III collagen.**

The Ehlers–Danlos syndromes (EDS) represent a clinically and genetically heterogeneous category of connective tissue disorders (1). Affected individuals have fragile connective tissue, bruise easily, and are loose-jointed. Seven types are separated on clinical, genetic, and biochemical grounds. Specific biochemical defects have been identified in two. Type VI has hydroxylysine-deficient collagen due to reduced lysyl hydroxylase levels (2–4). In type VII collagen precursors accumulate because of lowered procollagen peptidase activity (5). Both defects impair normal collagen crosslinking, producing fragile and hyperextensible connective tissues. In contrast to other forms, patients with type IV EDS have fragile but inextensible connective tissues (1). Rupture of large arteries and of the bowel are major catastrophes of this form of EDS. The patients' skin is unusually thin and shows a prominent venous network. Affected individuals show tight and thin skin over the face and ears. A remarkable clinical feature is a tendency to form keloids and contractures despite deficiency of collagen.

We have studied tissue and fibroblasts from five patients with EDS IV. Histologic and chemical studies show a reduced collagen content of skin and aorta. More significantly, there is a specific lack of type III collagen both in the tissues and in the proteins synthesized by cultured fibroblasts.

MATERIALS AND METHODS

Adequate specimens of skin, aorta, gut, bone, and tendon were obtained within 12 hr of the death of one patient. These were immediately frozen with dry ice and stored at −20° until studied. Skin biopsies were obtained from the left forearm of all other patients with a 4 mm punch. The samples were divided; part was stored at −20° and the remainder was donated to the American Type Culture Collection, Rockville, Md., for their Mutant Human Cell Bank. Various cell strains from the Bank were studied including presumed normals, EDS IV, and other EDS variants.

Control tissue was obtained within 24 hr of death from age- and sex-matched individuals and was stored at −20°. Portions of control and EDS IV tissues were fixed with formaldehyde, and paraffin-embedded sections were stained with hematoxylin and eosin, Masson's trichrome, and Weigert's elastic stain. The remainder was used for biochemical studies.

Biochemical studies

Amino-Acid Analysis. Weighed amounts of tissue were hydrolyzed in 6 N HCl at 107° for 24 hr under nitrogen. Amino-acid analyses were carried out by Mr. Guy Hawkins, Laboratory of Biochemistry, National Institute of Dental Research, using automatic equipment according to the method of Miller and Piez (6).

Cyanogen Bromide Digestion. Whole tissue and purified collagenous material were digested in 70% formic acid with a 2-fold excess of cyanogen bromide at 30° for 4 hr (7). The reaction mixture was diluted 10-fold with water and lyophilized to remove volatile components. Peptides produced by cyanogen bromide digestion were resolved by electrophoresis on 7.5% polyacrylamide gels in sodium dodecyl sulfate (8). Gels were stained for 45 min in Coomassie blue and destained in 7% acetic acid with 5% methanol.

Collagen Standards. Type I collagen was obtained from lathyritic rat skins and from human skin. Human type III collagen was solubilized by incubating fetal skin with pepsin at 15° for 4 hr (9–11). Type III collagen was precipitated with 1.5 M NaCl, 0.05 M Tris·HCl, pH 7.4, and subsequently purified by CM-cellulose chromatography (9–11). The supernatant fluid from the 1.5 M NaCl precipitate contained type I collagen.

Characterization of Collagenous Proteins Synthesized by Cultured Cells. Cells obtained from the American Type Culture Collection were maintained as described earlier (12–14). When confluent, the cultures received 10 ml of the Dulbecco–Vogt medium lacking glycine, lysine, glutamate, and serum but supplemented with ascorbic acid (50 μg/ml) and β-aminopropionitrile (50 μg/ml). Twenty microcuries each of [*U*-^{14}C]-glycine, [*U*-^{14}C]lysine, and [*U*-^{14}C]proline were added to each flask. Twenty-four hours later media and cell samples were collected. Collagenous proteins were precipitated from the media with 20% saturated $(NH_4)_2SO_4$ (15) and the precipitated proteins were dialyzed against the buffer used for separation of type I from type III procollagens by chromatography on DEAE-cellulose (13, 15). Alternatively, medium and cell layer were combined and exposed to pepsin in 0.5 M acetic

Abbreviation: EDS, Ehlers–Danlos syndrome.

TABLE 1. *Amino-acid composition of aortic tissue from an Ehlers–Danlos type IV patient and controls*

	EDS IV*	Control†
4-Hydroxyproline	10	30
Aspartic Acid	47	56
Threonine	26	29
Serine	22	34
Glutamic Acid	56	76
Proline	93	91
Glycine	226	236
Alanine	168	131
½ Cystine	6	13
Valine	117	89
Methionine	2.2	3.6
Isoleucine	21	25
Leucine	76	53
Tyrosine	22	18
Phenylalanine	37	24
Hydroxylysine	0.5	3.3
Histidine	17	14
Lysine	36	32
Arginine	23	35

Results are expressed in terms of residues per 1000 residues.
* Average of two analyses from one patient.
† Average of five analyses from five age- and sex-matched controls.

acid at 15° for 6 hr. Subsequently, type I and type III collagen chains were separated by CM-cellulose chromatography (9–11).

RESULTS

Differences in composition

Table 1 shows the amino acid composition of an EDS IV aorta compared with the average composition observed in five age- and sex-matched controls. There are several obvious differences. The EDS aorta contains much less hydroxyproline and hydroxylysine than does that of controls. The amounts of glycine, lysine, and proline, on the other hand, are similar. There is more valine in the EDS IV tissue. Most of the protein of ascending aorta is collagen and elastin (16). The analyses presented here suggest that EDS IV aorta contains much less collagen than normal control aortas, as indicated by the lowered levels of hydroxylysine and hydroxyproline. The high contents of valine and glycine relative to hydroxyproline suggest that elastin forms the major part of the EDS IV aorta. Histologic studies confirm that there are reduced amounts of collagen in the dermis, aorta, and submucosa of gut. Additionally, there is a reduction by about one-half in the size of individual collagen bundles.

Differences in the peptides derived by cyanogen bromide cleavage of tissues

Consistent differences were observed in the peptides obtained by cyanogen bromide cleavage of skin and aortas of normal controls as compared with EDS IV tissue. A peptide smaller than α1(I)-CB 3 was present in controls, but absent in skin of EDS IV patients (Fig. 1). This was the case in five skin samples from EDS IV patients that we have examined. Furthermore, a similar peptide is missing from CNBr digests of the aorta, gut, and lungs of an EDS IV patient but present in similar organs from controls (not shown). Using other

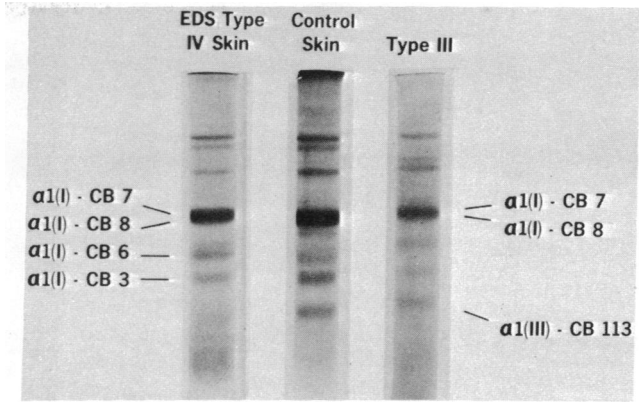

FIG. 1. Electrophoretic separation of the peptides produced by CNBr cleavage of control and EDS IV skin. A similar digest of authentic type III collagen is shown on the right. The nomenclature of Epstein (10) is used.

CNBr peptides from type I collagen as standards, the molecular weight of this peptide is estimated to be 10,000.

Electrophoresis of cyanogen bromide peptides from authentic human type III collagen showed a peptide migrating in this position. Epstein (10) and Chung *et al.* (17) have isolated similar peptides from human type III collagen, but their nomenclature differs. Epstein has called this peptide α1(III)-CB 113 because this component has approximately 113 amino acids, while Chung *et al.* (17) have called it α1(III)-CB 8. The electrophoretic migrations of authentic α1(III)-CB 113 and α1(III)-CB 8 exactly correspond to each other and to that of the CNBr peptide present in control tissue. These results indicate that type III collagen is absent from EDS type IV tissue.

This interpretation was strengthened by characterizing the collagens released by limited pepsin digestion from control and EDS type IV skin. In these studies, the material solubilized by pepsin was separated from the insoluble residue by centrifugation. The supernatant fluid was dialyzed against 1.5 M NaCl, 0.05 M Tris·HCl, pH 7.4. Material insoluble in this solvent was examined by electrophoresis (after denaturation) both with and without reduction with 2-mercaptoethanol. A high-molecular-weight (trimeric) component which was converted by reduction to α1 type chains was observed in control samples but not in EDS IV samples. Type III collagen is trimeric before reduction, but afterwards migrates as α1 type chains. These studies indicate that EDS IV skin lacks type III collagen. As suggested by the amino-acid analyses shown in Table 1, type I collagen is probably also reduced in EDS IV aorta.

Collagens synthesized by cultured control and EDS IV fibroblasts

Similar amounts of collagenous protein were synthesized by EDS IV and control cells. The collagens synthesized by cultured fibroblasts from EDS IV patients were compared with collagens from normal controls and patients with various inherited diseases. Cell strains from normal skin synthesize both type I and III collagens (15, 18). In culture, both of these proteins accumulate in the medium as higher-molecular-weight precursors, the procollagens (12, 13, 15, 18). Procollagen type I is readily resolved from procollagen type III by chromatography on DEAE-cellulose (13, 15, 18). In Fig. 2 the abnormal

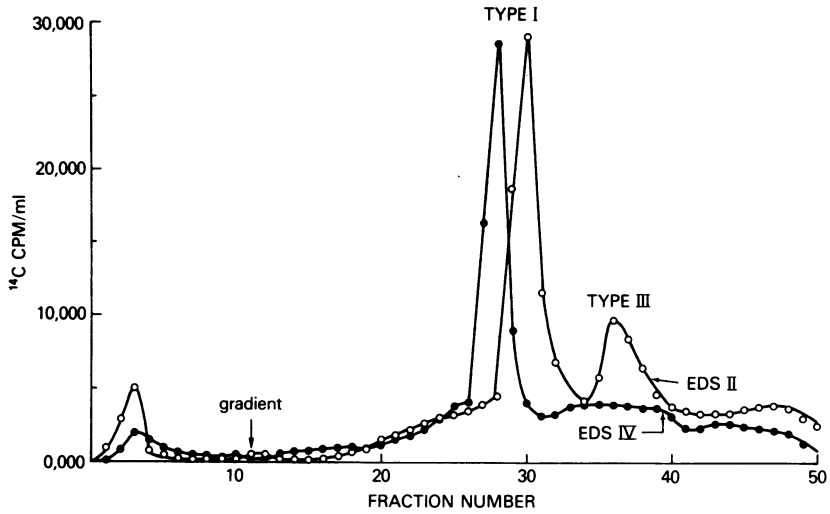

FIG. 2. DEAE-cellulose chromatography of the labeled proteins secreted by the fibroblasts into the medium. Proteins from an EDS IV patient are shown with solid circles, and proteins from an EDS II patient are shown with the open circles. The proteins from the two sources were chromatographed separately.

pattern of an EDS IV culture is contrasted with the normal pattern observed from an EDS II culture. Type III procollagen normally forms 6–20% of the total collagenous proteins secreted into the medium by normal cell lines. No type III procollagen was found to be synthesized by EDS IV cell lines. We studied cultured collagenous material by a limited cleavage of medium and cell collagenous protein with pepsin to dissolve insoluble collagen and convert precursor forms to collagen (14). Type I and type III chains were separated by standard chromatographic methods (9–11). Again EDS IV cell strains were found to lack type III collagen in comparison with control cell, EDS II, and EDS VI strains (not shown).

DISCUSSION

Miller *et al.* (7) found evidence for a second genetically distinct collagen in skin. Subsequently, this protein, now identified as type III collagen, was prepared from skin, intestine, and arteries. It is more plentiful in fetal than adult skin, but even in the adult it comprises 25–50% of total skin collagen (10).

EDS IV is a dangerously disabling disease with a particular liability to rupture of large arteries. In addition, the skin is thin and translucent and has a much reduced elasticity. We believe the deficiency of type III collagen observed in the skin and other tissue examined from EDS IV patients can explain many of the clinical abnormalities of this disorder. The amount of collagen was reduced especially in the aorta of patients, as evidenced by amino-acid analysis and light microscopic studies. Further, a peptide diagnostic for human type III collagen was absent from CNBr digests of skin, gut, and large arteries of EDS IV patients, but was present in tissues from controls and patients with EDS I, II, III, VI, the Marfan syndrome, and osteogenesis imperfecta (not shown). Correlating the pathologic manifestations of EDS IV with the biochemical findings, it seems likely that type III collagen is an essential component of distensible organs such as arteries. Its absence or reduction results in premature wear of such organs with resultant rupture in early adult life.

The absence of type III collagen in tissues is paralleled by a similar lack in cultured fibroblasts. None was detected in the cells or surrounding medium, suggesting that none was synthesized. Since the defect can be shown in fibroblasts, intrauterine diagnosis of the disease should now be possible. Ongoing studies of the parents of affected individuals show intermediate levels of type III collagens both in tissue and fibroblast culture and indicate that the inheritance of this disease is autosomal recessive. Very reduced amounts or absence of a structural protein in homozygotes and partially reduced levels in heterozygotes is reminiscent of other diseases such as the thalassemias. Similar defects in the synthesis of the other collagens may occur; indeed deficient type I collagen synthesis has been observed in patients with one form of osteogenesis imperfecta congenita (14).

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