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Processing of Types I and III Procollagen in Ehlers-Danlos Syndrome Type VII

RITVA HALILA,[1] BEAT STEINMANN,[2] AND LEENA PELTONEN[1]

SUMMARY

The processing of types I and III procollagen was studied in skin fibroblast cultures from type VII A and B of the Ehlers-Danlos syndrome [EDS] and age-matched controls. Synthesis of collagenous proteins was significantly increased in EDS type VII B, and the activities of prolyl-4-hydroxylase and galactosylhydroxylysyl glucosyltransferase were slightly increased in these cell lines, reflecting increased biosynthesis of collagen. The synthesis of collagenous proteins was close to normal in EDS type VII A cells. The synthesis of type III procollagen per cell was increased, as also was the ratio of immunoreactive type III procollagen to total collagen production. The activity of type I procollagen amino-terminal proteinase was decreased in skin fibroblasts of type VII A and normal in those of type VII B relative to cell protein or DNA. Type III amino-terminal proteinase activity was of a level found in normal cells when expressed relative to the protein or DNA, and the release of type III amino-terminal propeptides was nevertheless not disturbed in these EDS type VII cell cultures. The results show that only the conversion of type I procollagen is defective in EDS type VII, and no general defect in procollagen processing can be found in EDS type VII as has been suggested in the case of dermatosparaxis, a connective tissue disorder in animals caused by disturbed procollagen conversion.

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[1] Collagen Research Unit, University of Oulu, Department of Anatomy, SF-90220 Oulu 22, Finland. Present address: Recombinant-DNA Laboratory, University of Helsinki, Valimotie 7, 00380 Helsinki, Finland.

[2] Department of Pediatrics, University Hospital of Zurich, CH-8032 Zurich, Switzerland.

INTRODUCTION

The removal of the amino-terminal propeptides from procollagen types I-III is an extracellular event catalyzed by two specific enzymes: type I N-proteinase cleaving procollagens of types I and II, and type III N-proteinase, cleaving type III procollagen [1–4]. The removal of the N-terminal propeptide is believed to be a regulatory mechanism for fibril formation [5, 6], while the persistence of pN-collagen molecules in collagen fibrils seems to be a physiological phenomenon. pN-collagens of both type I and type III can be demonstrated in fetal skin, and type III pN-collagen molecules are still present in adult skin collagen fibers [7, 8]. Any predominance of these semiprocessed forms of collagen molecules in connective tissues, however, will result in disorders in which the clinical manifestations can be explained by thin, irregular collagen fibers that lack the normal physiological properties.

An accumulation of pN-collagen was first described in a disorder in cattle and sheep known as dermatosparaxis [9, 10], which is characterized by extreme skin fragility and caused by a deficiency in type I procollagen N-proteinase [9–11]. Impaired cleavage of the amino-terminal propeptide from type I procollagen in man results in type VII of the Ehlers-Danlos syndrome (EDS), a disorder characterized by extreme joint laxity and congenital bilateral hip dislocations with no significant skin fragility [12]. This syndrome has subsequently been divided into two subtypes: EDS type VII A, inherited probably as an autosomal recessive trait, in which the biochemical defect lies in a partial deficiency in type I procollagen N-proteinase activity [12, 13], and type VII B, inherited as an autosomal dominant condition, in which the structure of procollagen type I is altered due to a structural mutation in the proα2(I) collagen chain close to the cleavage site for type I N-proteinase [13, 15], thus preventing removal of type I procollagen N-propeptide.

It is not known as yet whether the processing of other collagen types is affected in these inherited disorders. Since defective processing of type III procollagen is also observable in the skin of dermatosparactic animals [4], and a more general endoglycosidase deficiency has recently been suggested as a primary cause of dermatosparaxis in animals [14], we set out here to study the amino-terminal processing of both type I and type III procollagen in skin fibroblast cultures from EDS patients representing the two types described above.

MATERIALS AND METHODS

Cell Cultures

Skin fibroblasts from the patients concerned were obtained from the American Type Culture Collection. Five lines (CRL 1148, CRL 1149, CRL 1150, CRL 1193, and CRL 1274) have been previously described to have a partial defect in the activity of type I procollagen amino-terminal proteinase [12, 13], and two lines (type VII B), a structural mutation of the procollagen proα2(I) chain [13, 15]. Normal cell lines were obtained from skin biopsies from age-matched healthy volunteers or taken from children during therapeutic operations.

The cells were grown in 150-mm^2 plastic flasks (Falcon) or in Petri dishes (diameter 90

mm) in Dulbecco's Modified Eagle's Medium containing 10% newborn calf serum, glutamine (290 μg/ml), ascorbate (50 μg/ml), streptomycin (100 μg/ml), and penicillin (100 U/ml). The cells were allowed to grow in a humidified atmosphere containing 5% CO_2 and 95% air in a Forma 3325 incubator. The medium was changed twice a week. Mycoplasma contamination was tested every month by staining the cytoplasmic DNA by the fluorometric method of Chen [16] and was found to be absent during the whole study.

Radioactive Labeling of the Cells

Fresh medium was always provided for cells 24 hrs prior to the labeling. After washing the cells twice with 2 ml of Dulbecco's phosphate-buffered saline (PBS), the labeling medium, containing 2.5 μCi/ml of [^{14}C]proline, 5% dialyzed fetal calf serum, glutamine, ascorbate, and antibiotics in the concentrations described above, was added to the cultures. After 24 hrs labeling, the cell cultures were placed on an ice bath, the medium was removed, and proteinase inhibitors were added to final concentrations of 25 mM EDTA, 1 mM PABA, 1 mM PMSF, and 10 mM NEM. The cells were then washed with 1 ml of Dulbecco's PBS and this medium pooled with that removed earlier. The cells were scraped with a rubber policeman into 0.4 M NaCl and 0.1% Triton X-100 in 0.1 M Tris-HCl, pH 7.4, containing the proteinase inhibitors in the same concentrations as in the medium collected. The medium proteins were further precipitated by adding 390 mg/ml of solid ammonium sulfate, precipitation was allowed to proceed overnight, and the medium was centrifuged at 11,000 *g* for 1 hr. The pellet was suspended in 0.5 ml of 0.4 M NaCl in 0.1 M Tris-HCl, pH 7.4, and dialyzed against the same buffer. One hundred microliters of this sample was taken for determination of [^{14}C]hydroxyproline, and the rest was heated in SDS gel sample buffer containing 2% SDS, 10% glycerol, and 0.001% bromophenol blue in 0.125% Tris-HCl, pH 6.8, and dialyzed against the same buffer.

Determination of Procollagen Synthesis

One hundred microliters of 12 N HCl was added to an equal volume of the [^{14}C]proline labeling sample described above and hydrolyzed at 2 atm and 116°C for 16 hrs. The amounts of [^{14}C]hydroxyproline and [^{14}C]proline were analyzed as described [17].

Determination of the Activity of Type III Procollagen N-Proteinase

The method was based on sequential ammonium sulfate precipitation of the pN-collagen substrate and cleavage products, as described [18]. Briefly, the cells were extracted and homogenized in 2 M KCl and 0.1% Triton X-100 in 0.05 M sodium cacodylate buffer, pH 7.4. After stirring for 4 hrs, the homogenate was centrifuged at 11,000 *g* for 1 hr and the supernatant recovered. The pellet was then resuspended in the same buffer, homogenized, and centrifuged, and this supernatant was combined with the first. The excess salt was then removed by stepwise dialysis of the extract against 1 M KCl-0.5 M KCl-0.2 M KCl-0.1 M KCl in 0.05 M sodium cacodylate buffer, pH 7.4. Ten to fifty microliters of the cell extract was assayed for enzyme activity in the presence of 10–20 μg of [^{14}C]carboxymethylated calf type III pN-collagen, 0.15 M NaCl, 2 mM $CaCl_2$, 1 mM of PMSF, and 10 mM of NEM in a total volume of 140 μl. After incubation for 4 hrs, the reaction was stopped by cooling the assay tubes in an ice bath and adding 20 μl of a mixture containing 250 mM sodium EDTA, 10 mM PABA, 10 mM PMSF, and 100 mM NEM, 40 μl of newborn calf serum, and 100 μl of 90% ammonium sulfate in 0.1 M sodium cacodylate buffer, pH 7.4. The ammonium sulfate precipitation was allowed to proceed for 2–4 hrs at 4°C, and the samples were then centrifuged at 12,000 *g* for 15 min. The activity of the enzyme was detected as radioactivity released into the supernatant. Every assay had a control with all the reagents present except for the cell extract.

Determination of the Activity of Type I Procollagen N-Proteinase

This method has been described by Tuderman et al. [1, 2]. Our assay used [^{14}C]carboxymethylated type I pN-collagen extracted from fetal calf skin as the substrate. The total assay volume was 140 μl, and precipitation was carried out as described for type III N-proteinase [18].

Assay of Type III Procollagen Amino-Terminal Propeptide

The cells were cultured as described above, the medium was removed, and the cells were washed once with PBS. After 24 hrs incubation in normal cell culture conditions, the cells were placed on an ice bath, the medium was removed, and the cells were washed with 1 ml of Dulbecco's PBS, which was then combined with the medium removed. This solution was frozen in small aliquots at −70°C for further use. The medium was then assayed for type III N-propeptide with a commercial radioimmunoassay (RIA) kit (Hoechst) for this peptide following the manufacturer's instructions [19]. An aliquot of the media collected were chromatographed on Ultrogel AcA 44 (1.6 × 82 cm), and fractions of 2 ml were collected. The proportions of free and bound amino-terminal propeptides were measured by radioimmunoassay, and the elution profile was compared with the elution points of the globular protein standards IgG, bovine serum albumin, ovalbumin, and soybean trypsin inhibitor.

Other Assays

Polyacrylamide slab gel electrophoresis was carried out as described by King and Laemmli [20]. After electrophoresis, the gels were impregnated with Me_2SO-PPO, dried under vacuum, and analyzed by scanning densitometry [21, 22]. The activities of prolyl-4-hydroxylase and galactosylhydroxylysyl glucosyltransferase were assayed as described by Kivirikko and Myllylä [23]. The cells were counted in a Bürger-Türk hemocytometer. DNA in the cells from the cell extracts was determined by the method of Brunk et al. [24], and the total protein concentration was measured using Bio-Rad protein assay kit (Bio Rad, Richmond, Calif.).

RESULTS

Production of Collagenous Proteins by EDS Type VII Fibroblasts

Total procollagen production per cell, measured as [^{14}C]hydroxyproline incorporation in 24 hrs was found to be twice as great in EDS type VII B fibroblast cell lines as in cells from age-matched controls (table 1), an effect that was also obvious from the ratio of hydroxyproline to proline, the mean of which was 1.3 times greater in the EDS type VII B fibroblast cultures than in the control cultures (table 1). The incorporation of [^{14}C]hydroxyproline in the EDS type VII A cultures did not differ significantly from that in the controls. There was no difference between the two cell lines of EDS type VII B either in the incorporation of [^{14}C]hydroxyproline, in the pattern of [^{14}C]-labeled proteins in SDS gel electrophoresis, and it seems on the basis of pepsin digestions, two-dimensional SDS-polyacrylamide gel electrophoresis of CNBr-peptides, and the present results that the molecular defect in the patient originating from Libya is identical to that in the patient previously reported from the U.S. to have EDS type VII B [13, 15].

The proportion of the total production of collagenous proteins accounted for by type III procollagen was determined by assaying the ratio of immuno-

TABLE 1

PROCOLLAGEN PRODUCTION IN FIBROBLAST CULTURES FROM PATIENTS WITH EDS TYPE VII

Cell line	cpm [^{14}C]hyp*/10^{6}cells	cpm [^{14}C]pro†/10^{6}cells	Hyp/pro
EDS type VII A (n = 5).....	19,582 ± 3,000	96,791 ± 20,000	0.20 ± 0.03
EDS type VII B (n = 2).....	38,296 ± 4,400	132,137 ± 15,000	0.29 ± 0.01
Age-matched controls (n = 6).....	25,282 ± 4,800	116,214 ± 12,000	0.22 ± 0.03
Other controls (n = 9).....	16,557 ± 2,200	76,116 ± 9,900	0.22 ± 0.04

NOTE: The confluent fibroblast cultures were labeled with 2.5 μCi/ml of [^{14}C]proline for 24 hrs (n = no. cell lines). The proteins in the culture medium and the cells were analyzed for protein incorporated [^{14}C] hydroxyproline and proline as described in MATERIALS AND METHODS [17]. Every cell line was assayed for hydroxyproline at least twice. Mean values for these determinations are shown in the table.

* hyp = hydroxyproline.

† pro = proline.

reactive type III procollagen N-propeptide to protein incorporated [^{14}C]hydroxyproline in 24-hr medium (table 2). Both EDS type VII A and B cell cultures demonstrated a significant increase in the production of type III procollagen to three to four times that of the controls when expressed per cell and the ratio of type III amino-terminal propeptide to total incorporation of [^{14}C]hydroxyproline was also altered in both cell lines (table 2). Since the radioimmunoassay for type III procollagen amino-terminal propeptide quantifies not only free amino-terminal propeptides but also those still attached to collagen molecules (type III procollagen and type III pN-collagen), a closer identification of the molecules detected with this radioimmunoassay was attempted by molecular sieve chromatography (Ultrogel AcA 44), in which type III procollagen (mol. wt. 360,000) and type III pN-collagen (mol. wt. 330,000) can be separated from free type III amino-terminal propeptide (mol. wt. 56,000) (fig. 1, see below).

TABLE 2

AMOUNTS OF TYPE III PROCOLLAGEN AMINO-TERMINAL PROPEPTIDE IN THE MEDIUM AFTER 24 HRS

	III N-propeptide	
CELL LINE	ng/10^{6}cells	ng/cpm hyp*
EDS type VII A (n = 5) ...	1,641 ± 280	0.084 ± 0.004
EDS type VII B (n = 2) ...	2,420 ± 730	0.063 ± 0.006
Controls (n = 15)	557 ± 310	0.034 ± 0.005

NOTE: Type III amino-terminal propeptide was determined from the 24-hr medium of fibroblast cultures (n = no. cell lines) according to the instructions given by the manufacturer of the kit (see MATERIALS AND METHODS) at dilutions of 1:40-1:1,000 in PBS. At least four dilutions were tested in order to achieve 50% inhibition. Control cultures were not divided into age-matched and other control cultures, while such variation in these cultures were not recorded.

* = hydroxyproline.

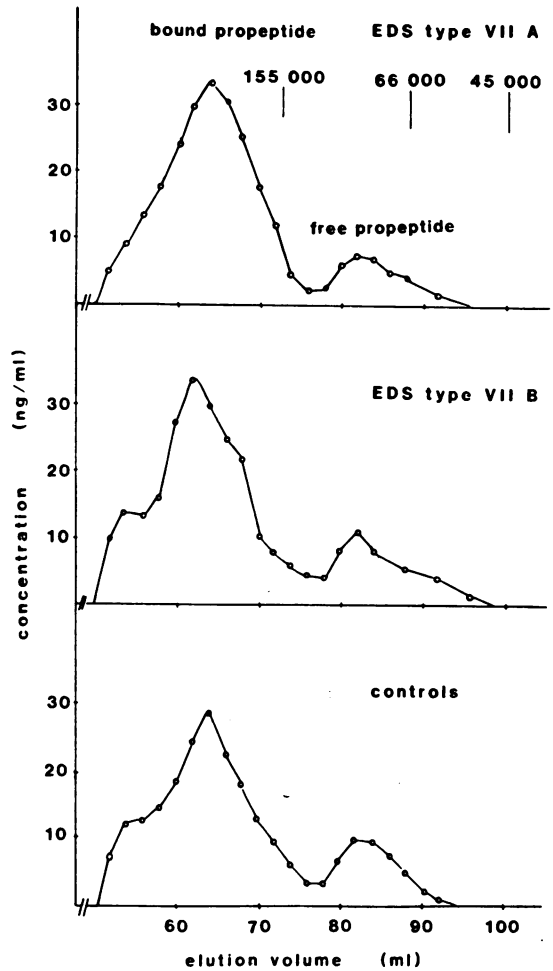

FIG. 1.—Processing of type III procollagen in EDS type VII fibroblast culture medium. An aliquot of the 24-hr medium containing always the same amount of type III N-propeptides was passed through Ultrogel AcA 44 as described in MATERIALS AND METHODS. The fractions of 2 ml were collected, and fractions 26–60 were assayed for the concentration of type III amino-terminal propeptide by a commercial RIA kit (Hoechst). The migration of the peaks have been compared with migration positions of globular standards (IgG, bovine serum albumin, ovalbumin, and soybean trypsin inhibitor). The elution of free propeptide and type III collagen-bound propeptide have been marked in the figure.

Activity of Amino-Terminal Proteinases in EDS Type VII Fibroblasts

Assays of the activity of types I and III amino-terminal proteinases from the confluent fibroblast cultures showed the activity of type I amino-terminal proteinase per μg of protein or DNA to be less than 50% of the control value in EDS type VII A fibroblast cultures, confirming the results of Steinmann et al. [13], whereas the cell extracts from EDS type VII B did not show any decrease

TABLE 3

ACTIVITY OF TYPE I AND III PROCOLLAGEN N-PROTEINASES IN EDS TYPE VII

	TYPE I		TYPE III	
CELL LINE	/μg prot	/μg DNA	/μg prot	/μg DNA
EDS type VII A (n = 5)	4.14	440	6.28	1,261
EDS type VII B (n = 2)	8.59	796	4.77	1,041
Controls (n = 12)	9.35	1,091	5.15	1,071

NOTE: The activity of the type I and type III procollagen amino-terminal proteinases was determined as described in MATERIALS AND METHODS. Every cell line (no. expressed in parentheses) was assayed at least 16 times for enzyme activities. Means for these activities are shown in the table. The variations between parallel assays have been described [18].

in the type I procollagen type I N-proteinase activity (table 3). The activity of type III N-proteinase was not significantly altered in either the type VII A or the type B EDS cell cultures (table 3).

In order to confirm that the decreased N-proteinase activities in the EDS cell cultures were not merely cases of nonspecific variation in collagen biosynthesis in these cell lines, we assayed the activities of prolyl-4-hydroxylase (PH) and galactosylhydroxylysyl glucosyltransferase (GGT). These did not differ from the controls in type VII A, but were increased when expressed relative to μg of protein or DNA, as expected, in the type VII B cell lines, where there was increased incorporation of [^{14}C]hydroxyproline (table 4).

Conversion of Procollagen in EDS Type VII Fibroblast Cultures

In order to determine whether the lower activity of type III procollagen amino-terminal proteinase in relative terms in the EDS type VII cells disturbed the conversion of this procollagen type in cell cultures, an assay was made of the relative amounts of the type III procollagen N-propeptide released in control and EDS type VII cell cultures after separation of the propeptide from pN-collagen and type III procollagen molecules by molecular sieve chromatog-

TABLE 4

ACTIVITY OF PROLYL HYDROXYLASE (PH) AND GALACTOSYLHYDROXYLYSYL GLUCOSYLTRANSFERASE (GGT) IN EDS TYPE VII

	PH			GGT		
CELL LINE	/μg prot	/μg DNA	/hyp*	/μg prot	/μg DNA	/hyp
EDS type VII A (n = 5) ...	1.19	60.6	4.57	32.2	1,580	1.11
EDS type VII B (n = 2) ...	2.93	104.6	2.83	54.0	1,980	0.57
Controls (n = 7)	1.31	55.2	4.35	30.3	1,220	1.81

NOTE: Cell extracts from the fibroblast cultures were assayed for prolyl-4-hydroxylase and galactosylhydroxylysyl glucosyltransferase as described [26]. The cells (n = no. cell lines) were extracted in 0.4 M NaCl and 0.1 % Triton X-100 in 0.1 M Tris-HCl, pH 7.4. After stirring, they were centrifuged and the enzyme activities measured from the supernatant at two concentrations of the enzyme preparation. The means of the two assays are shown in the table.

* hyp = hydroxyproline.

TABLE 5

PROPORTIONS OF FREE AND UNCLEAVED TYPE III N-PROPEPTIDE IN EDS TYPE VII

CELL LINE	TYPE III PROCOLLAGEN N-PROPEPTIDE	
	Free %	Bound %
EDS type VII A (n = 2)	22.5	77.5
EDS type VII B (n = 2)	25.6	74.4
Controls (n = 4)	25.0	75.0

NOTE: Part of the medium of the fibroblast cultures was passed through an Ultrogel AcA-44 column (1.5 × 82 cm) and fractions of 2 ml collected. Fractions 26–60 were assayed for type III amino-terminal propeptide. The ratio of free to collagen bound propeptide is expressed as a percentage of the total amount of propeptide detected in gel filtration.

raphy. As shown in figure 1 and table 5, the amino-terminal conversion of type III procollagen during 24 hrs was not significantly disturbed in either type VII A or type VII B EDS cell cultures, the proportion of free type III N-propeptide being close to the control values in both cases (fig. 1 and table 5).

The question of whether the increased production of collagen by the EDS type VII B cell lines was due to a lack of inhibition of collagen biosynthesis by the N-propeptides released was examined by adding fetal calf type I pN-collagen to the culture medium of these cells. After 24 hrs preincubation, the cells were labeled with 2.5 μCi/ml of [^{14}C]proline, and the production of collagenous proteins was determined by measuring protein-incorporated [^{14}C]hydroxyproline (see MATERIALS AND METHODS) for 24 hrs. As shown in table 6, no decrease in the incorporation of [^{14}C]hydroxyproline could be detected when compared with the cultures without additional type I pN-collagen molecules. The cleavage of this pN-collagen was confirmed by adding 20 μg/ml of [^{14}C]carboxymethylated type I pN-collagen to parallel cultures and monitoring the cleavage of this additional pN-collagen by SDS polyacrylamide gel electrophoresis. The pN-collagen was processed to collagen as well in EDS type VII B cell cultures as in control cell cultures (not shown).

DISCUSSION

The results presented here confirm the significant decrease in the activity of type I amino-terminal proteinase in cell cultures from EDS type VII A patients [12, 13]. This is the first time that this activity has been assayed and expressed quantitatively in a series of EDS type VII A fibroblast cultures and compared with those from patients with EDS type VII B and controls. The activity of type I amino-terminal proteinase in EDS type VII B fibroblasts was not lower than in the controls when expressed relative to the DNA content of the cells, this finding being in agreement with previous results suggesting normal enzyme activity in one EDS type VII B cell line [13]. In the case of type III N-proteinase, the reduced enzyme/substrate ratio did not affect the amino-terminal processing of type III procollagen. The processing of this procollagen type is known to be slow compared to that of type I procollagen [25, 26] and

TABLE 6

EFFECT OF ADDED CALF TYPE I pN-COLLAGEN ON THE PRODUCTION OF COLLAGENOUS PROTEINS IN EDS TYPE VII B

	TYPE I pN-COLLAGEN			
	ADDED		NOT ADDED	
CELL LINE	[^{14}C]hyp*/10^{6}cells	Hyp/pro†	[^{14}C]Hyp/10^{6}cells	Hyp/pro
EDS type VII B (n = 2)	39,388	0.29	38,296	0.29
Controls (n = 2)	29,370	0.20	26,365	0.20

NOTE: 20 μg/ml of type I pN-collagen was added to each confluent fibroblast culture. The cells were washed and the medium changed after 24 hrs, and the same amount of pN-collagen was again added. The cells were labeled for 24 hrs with [^{14}C]proline and the amount of protein incorporated [^{14}C]hydroxyproline assayed as in table 1 [17]. Parallel cultures were assayed under the same conditions but without any added type I pN-collagen.

* hyp = hydroxyproline.

† pro = proline.

incomplete even at tissue level [7]. It seems thus possible that the relative reduction in the activity of type III N-proteinase does not play as important a role in vivo as that of type I N-proteinase.

The increased incorporation of [^{14}C]hydroxyproline in the EDS type VII B fibroblast cultures is an interesting phenomenon for cell cultures in which the amino-terminal processing of type I procollagen to collagen has been shown to be defective, resulting in a decrease in free propeptides. It has been speculated that these propeptides may function as feedback inhibitors of collagen biosynthesis [27, 28]. As added fetal calf type I pN-collagen did not reduce the incorporation of [^{14}C]hydroxyproline during the following 24 hrs to any extent, we conclude that this hypothesis is not supported by our results even though the production of collagenous proteins is shown to be increased in these cell lines.

Previous studies have demonstrated a glycosylation defect in the amino-terminal propeptide of type III procollagen chains and suggest a more general glycosylation defect as the basic feature of the animal disorder dermatosparaxis [14], which is considered biochemically analog to the human EDS type VII [29]. According to this hypothesis, the processing of type III procollagen by a separate enzyme, type III amino-terminal proteinase, would also be disturbed. The activity of type III N-proteinase was not significantly lower than in controls in either type of EDS fibroblast culture, neither did the ratio of released type III amino-terminal propeptides to unprocessed type III procollagen of pN-collagen molecules differ from that in the control or EDS type VII A fibroblast cultures. These results provide no evidence for any generalized defect also affecting type III procollagen or the enzymes converting this molecule.

The connective tissue disorders EDS type VII in man and dermatosparaxis in many animals have been shown to have a similar type of defect in the conversion of procollagen type I to collagen [9–13, 30], but the different clinical manifestations, skin fragility in dermatosparaxis and extreme joint laxity in man, raise doubts about the identity of these syndromes. The activity of type I procollagen N-proteinase has been observed to be reduced in dermatosparaxis [8, 30], as has also been shown in the case of EDS type VII A [12]. A general-

ized glycosylation defect affecting both procollagens and their converting enzymes has nevertheless been suggested as a primary cause for the disturbed conversion in dermatosparaxis [14], and some indirect evidence for disturbed conversion of type III procollagen to collagen in this disorder has also been put forward [4]. Our results regarding human EDS type VII fibroblast cultures suggest that no defect in the conversion of type III procollagen to collagen can be demonstrated, a finding that excludes any generalized disturbance in the processing of procollagens and suggests a difference in the basic biochemical defect in this human disorder from that causing dermatosparaxis in animals.

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