

# Evidence for a relationship between Ehlers-Danlos type VII C in humans and bovine dermatosparaxis

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Ehlers-Danlos (ED) syndrome type VII is characterized by the accumulation of collagen precursors in connective tissues. ED VII A and B are caused by mutations in the genes of  $\alpha 1$  and  $\alpha 2$  collagen I which result in the disruption of the cleavage site of procollagen I N-proteinase. The existence of ED VII C in humans has been hypothesized on the basis of a disorder in cattle and sheep related to the absence of the enzyme. We now present evidence for the existence of this disease in humans, characterized by skin fragility, altered polymers seen as hieroglyphic pictures with electron microscopy, accumulation of p-N- $\alpha 1$  and p-N- $\alpha 2$  collagen type I in the dermis and absence of processing of the p-N-I polypeptides in fibroblast cultures.

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Collagen type I, the most abundant protein in mammals, is synthesized in the form of a precursor molecule bearing N- and C-terminal peptides that are cleaved by specific endopeptidases, procollagen I N-proteinase and procollagen I C-proteinase (Fig. 1). Only the mature processed molecules are able to form the cylindrical fibrils, organized in bundles, responsible for the remarkable mechanical properties of connective tissues. The discovery of these post-translational modifications of collagen molecules was derived from the observation, in cattle of a procollagen I N-proteinase deficiency. This leads to the accumulation in connective tissue of partially processed collagen precursor molecules composed of  $\alpha 1$  and  $\alpha 2$  polypeptides extended at their N-terminal ends. This hereditary disorder, called dermatosparaxis, is characterized by an extreme fragility of the skin. Our findings provide evidence for the existence in humans of a procollagen I N-proteinase deficiency, a disorder similar to dermatosparaxis and classified as Ehlers-Danlos type VII C.

ED syndrome is a group of inherited connective tissue diseases characterized by altered rheological properties of skin, joints, blood vessels and fascia. The syndrome comprises at least nine subtypes defined on a clinical, genetic and, for some of them, biochemical basis. ED type VII is characterized by an accumulation in various connective tissues of N-terminal extended precursors of type I collagen pN- $\alpha 1$  and/or pN- $\alpha 2$ . Two forms of the disease have been substantiated<sup>1-4</sup>. ED VII A and VII B (ref. 5), are related to the absence of collagen type I pN- $\alpha 1$  and pN- $\alpha 2$  polypeptide processing, respectively. Both are due to splicing mutations<sup>6-8</sup> affecting the corresponding

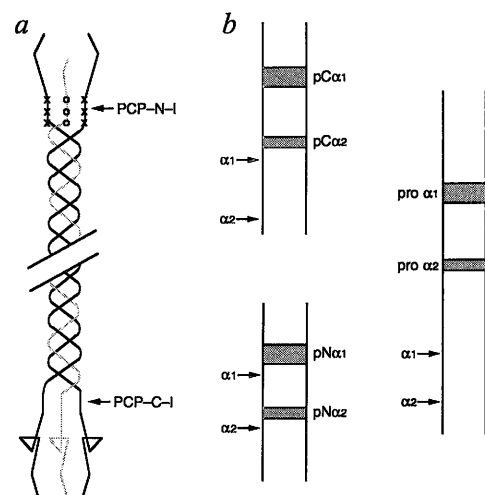


Fig. 1 Schematic representation of collagen type I precursor. a, Structure of collagen type I composed of two pro- $\alpha 1$  (solid line) and one pro- $\alpha 2$  (dotted line) chains. Two specific endopeptidases are responsible for removing the terminal propeptides, procollagen I carboxy-proteinase (PCP-C-I) and procollagen I amino-proteinase (PCP-N-I). b, SDS-PAGE representation of mutations suppressing the cleavage site of PCP-N-I in pro- $\alpha 1$  (xxx) or pro- $\alpha 2$  (ooo) responsible for, respectively, the form A and B of the ED syndrome type VII. Our patient presents the C form related to the inactivity of the PCP-N-I.  $\alpha 1$  and  $\alpha 2$  represent fully processed polypeptides; pC- $\alpha 1$  and pC- $\alpha 2$  are partially processed polypeptides still extended by the C-terminus, resulting from the PCP-N-I activity; pN- $\alpha 1$  and pN- $\alpha 2$  resulting from the PCP-C-I activity, and retain the N-terminal propeptides.

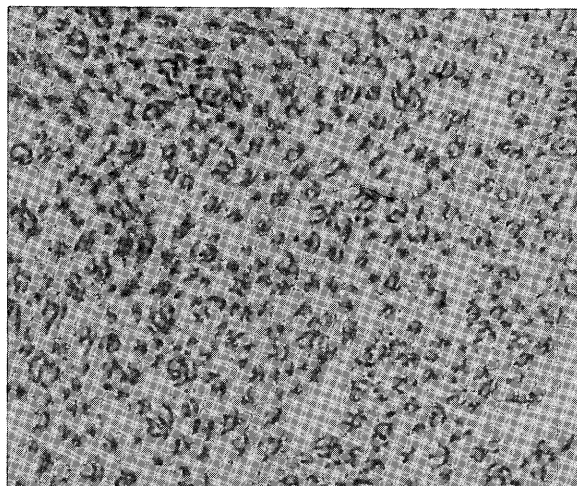


Fig. 2 Transmission electron microscopy of the dermis of the patient. The transverse section of bundles of collagen polymers illustrates the hieroglyphic pattern of individual fibrils embedded in an abundant amorphous ground substance as also described in bovine dermatosparaxis<sup>26,27</sup>. Magnification:  $\times 25,000$ . In longitudinal sections, the fibrils appear in the form of ribbons (not illustrated). The same pattern was observed in all parts of the dermis.

mRNAs by a deletion of the cleavage site (Fig. 1) for procollagen I N-proteinase (EC 3-4-24-14). A third form, ED VII C, has been postulated<sup>5,9,10</sup> on the basis of a heritable disease, dermatosparaxis, in cattle<sup>11,12</sup> and sheep<sup>13</sup> which is related to a defective activity of this specific endopeptidase. We now confirm the existence of ED VII C in humans and its similarity to dermatosparaxis.

#### Clinical signs

The patient is a two year-old girl and the first child of unrelated phenotypically normal parents (see

methodology for further details). Birth weight was 810 g and height 35 cm. Dysmorphic features included large appearing eyes and thickened eyelids. The postnatal evolution was uneventful. At 3 1/2 months, she was seen for small stature, dysmorphic facies and lack of ossification of cranial bones, weight 3.320 g (P10), height 47 cm (<P3) and head circumference 35.2 cm (P25). At the end of the first year, easy bruising developed and at 12 months of age, left occipital fracture with massive hematoma occurred following a minor fall. ED syndrome was then suspected on the basis of blue sclerae, soft, velvety and hyperextensible skin. Marked bruising and open wounds following minimal trauma were noted with developing motor activity of the child. Suturing was not possible due to the extreme fragility of the skin. Surgical removal of the large umbilical hernia was performed at the age of 20 months. The excised skin could be torn by hand. The clinical history and the symptoms in the patient differ from those observed in the ED VII A and B that are mostly characterized by joint laxity and congenital hip dislocations not present in our patient. The skin fragility in the patient is as severe as that observed in the dermatosparactic calves.

#### Collagen fibril ultrastructure

By electron microscopy (Fig. 2), the fibrils in cross section display the hieroglyphic figures typical of dermatosparactic skin of calves<sup>14</sup> and sheep<sup>13</sup>. They are strikingly different compared with the circular outline of collagen fibrils in normal skin. In longitudinal section, the ribbon-like structures have the characteristic 64 nm periodicity of type I collagen fibres (not shown). Such a pattern has not been illustrated in humans except for a recent abstract by Smith *et al.*<sup>15</sup>.

#### Collagen in the dermis

The concentration of collagen was lower in the skin of the patient ( $588 \text{ mg mg}^{-1}$  dry weight) compared with skin from normal age matched controls ( $704 \pm 37 \text{ mg mg}^{-1}$  dry weight,  $n = 4$ ). The extractability of collagen under nondenaturing conditions in neutral buffer and in 0.5M acetic acid was twice as high as controls. This might be explained by a defective formation of the stable intermolecular crosslinks, as observed in dermatosparaxis<sup>16</sup>.

The pattern of extracted collagen polypeptides from the skin specimen was similar to that observed in dermatosparactic skin (Fig. 3a). It is composed of a large proportion of pN- $\alpha 1$  and pN- $\alpha 2$  chains in the usual 2:1 ratio and a small portion of completely processed  $\alpha$ -chains.

#### p-N-I collagen processing *in vitro*

Incubation of the collagen extracted from the skin of the patient in the presence of a purified preparation of

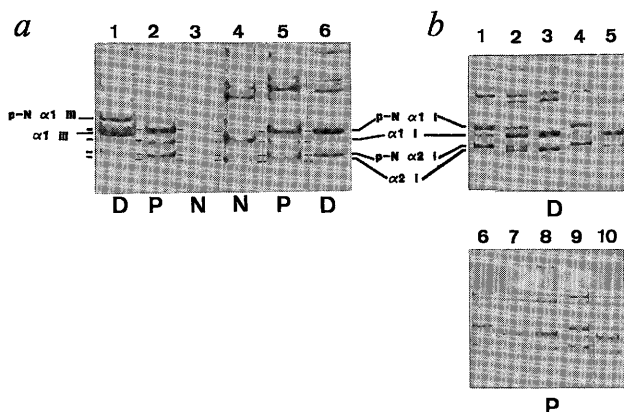


Fig. 3 a, Electrophoretic pattern of the collagen polypeptides extracted from the skin of the patient (P), compared to normal human skin (N) and dermatosparactic calf skin (D). Lanes 1-3, 0.15M NaCl extracts analysed under reducing conditions. Lanes 4-6, 0.5M acetic acid extracts analysed under non-reducing conditions. The pattern of the polypeptides in the patient (lane 2 and 5) resembles that of the collagen extracted from dermatosparactic skin (lanes 1 and 6) by the presence of the N-terminal extended precursor of  $\alpha 1$  (p-N- $\alpha 1$ ) and  $\alpha 2$  (p-N- $\alpha 2$ ) not found in normal skin (lanes 3 and 4). b, Procollagen I N-proteinase and pepsin proteolysis of 0.5 M acetic acid extracted collagen from dermatosparactic calf skin (lanes 1-5) and from the skin of the patient (lanes 6-10). The samples were incubated with purified procollagen I N-proteinase (PCP) or with pepsin, precipitated at 50% ethanol and analysed by SDS-PAGE as in a. Lanes 1 and 6, untreated; lanes 2 and 7, 4 units of PCP; lanes 3 and 8, 40 units of PCP; lanes 4 and 9, 40 units of PCP in presence of 80 mM EDTA; lanes 5 and 10, 2.5  $\mu\text{g}$  of pepsin. The processing of the pN  $\alpha 1$  and pN  $\alpha 2$  polypeptides occurs similarly and is complete upon incubation with 40 units of procollagen N-proteinase or 2.5  $\mu\text{g}$  of pepsin. Procollagen N-proteinase is inhibited by chelation of calcium<sup>20</sup>.

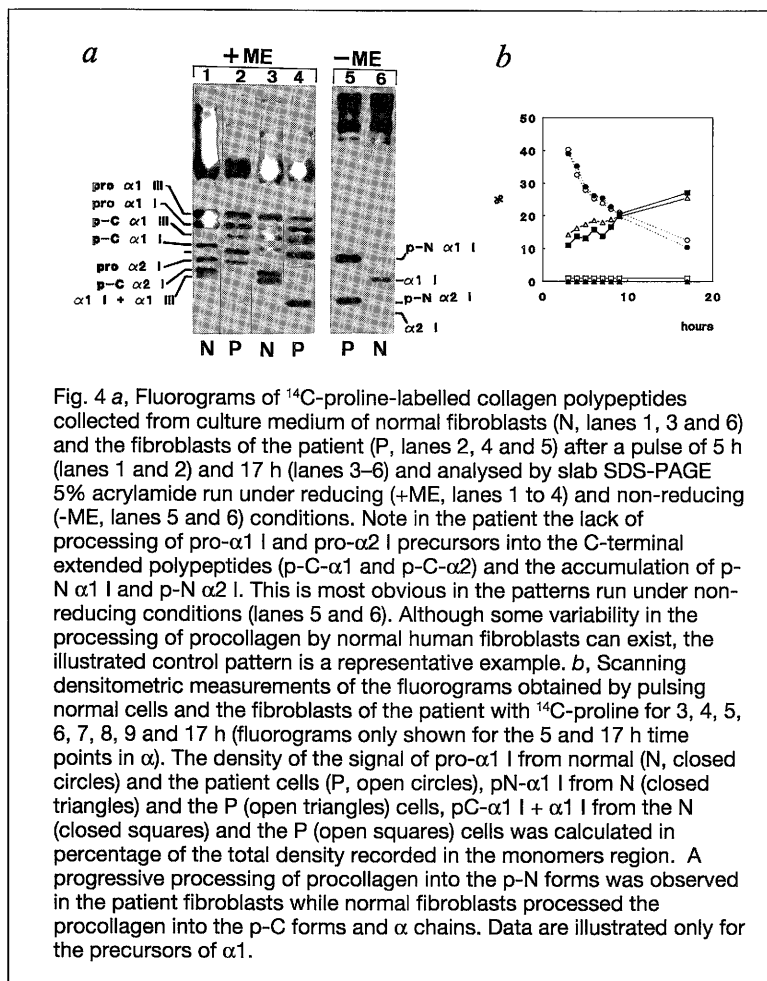


Fig. 4 a, Fluorograms of <sup>14</sup>C-proline-labelled collagen polypeptides collected from culture medium of normal fibroblasts (N, lanes 1, 3 and 6) and the fibroblasts of the patient (P, lanes 2, 4 and 5) after a pulse of 5 h (lanes 1 and 2) and 17 h (lanes 3–6) and analysed by slab SDS-PAGE 5% acrylamide run under reducing (+ME, lanes 1 to 4) and non-reducing (-ME, lanes 5 and 6) conditions. Note in the patient the lack of processing of pro-α1 I and pro-α2 I precursors into the C-terminal extended polypeptides (p-C-α1 and p-C-α2) and the accumulation of p-N α1 I and p-N α2 I. This is most obvious in the patterns run under non-reducing conditions (lanes 5 and 6). Although some variability in the processing of procollagen by normal human fibroblasts can exist, the illustrated control pattern is a representative example. b, Scanning densitometric measurements of the fluorograms obtained by pulsing normal cells and the fibroblasts of the patient with <sup>14</sup>C-proline for 3, 4, 5, 6, 7, 8, 9 and 17 h (fluorograms only shown for the 5 and 17 h time points in a). The density of the signal of pro-α1 I from normal (N, closed circles) and the patient cells (P, open circles), pN-α1 I from N (closed triangles) and the P (open triangles) cells, pC-α1 I + α1 I from the N (closed squares) and the P (open squares) cells was calculated in percentage of the total density recorded in the monomers region. A progressive processing of procollagen into the p-N forms was observed in the patient fibroblasts while normal fibroblasts processed the procollagen into the p-C forms and α chains. Data are illustrated only for the precursors of α1.

procollagen I N-proteinase or pepsin (Fig. 3b) resulted in the conversion of the p-N-I polypeptides to fully processed α chains as also observed in dermatosparaxis. These findings are in marked contrast to what is observed in ED VII types A and B, in which the absence of the cleavage site for the enzyme is responsible for the lack of activity of procollagen I N-proteinase and pepsin<sup>4,17</sup>.

#### p-N-I collagen accumulates in fibroblasts cultures

Skin fibroblasts cultures of the patient were established and labelled with <sup>14</sup>C- or <sup>3</sup>H-proline. Collagen synthesis measured by the incorporation of labelled proline and its conversion to <sup>3</sup>H-hydroxyproline<sup>18</sup> was at least twice as high in the fibroblasts of the patient ( $184.3 \pm 3.6 \times 10^3$  cpm  $\mu\text{g}^{-1}$  DNA) compared with skin cells from two age matched controls ( $79.3 \pm 6.5$  and  $77.8 \pm 0.6 \times 10^3$  cpm  $\mu\text{g}^{-1}$  DNA). The proportion of collagen in the newly synthesized proteins in culture was also increased (10.8% as compared to 7.4 and 7.7% in the two strains of control fibroblasts). The loss of a negative feed-back control by the released N-terminal peptides on collagen synthesis<sup>19</sup> might explain this increased collagen synthetic activity. This is also observed in the fibroblasts of dermatosparactic animals. As illustrated in Fig. 4a, the electrophoretic pattern of <sup>14</sup>C-labelled collagen polypeptides from the patients fibroblasts revealed a lack of processing of pro-α1 (I) and pro-α2 (I) into their p-C intermediates, but an accumulation of the p-N-precursor forms (Fig. 4b). These data demonstrate the lack of activity of the procollagen I N-proteinase while

the procollagen I C-proteinase is fully active.

#### Discussion

Procollagen I N-proteinase can be extracted from tissues<sup>20</sup> but has so far escaped complete purification and cloning. It is thought to be a multiple subunit enzyme complex<sup>21</sup>. Absence of collagen binding activity (anchorin) has been demonstrated in dermatosparactic sheep<sup>22</sup>. It is possible that defective processing of procollagen, as in dermatosparaxis and in the ED type VII C, results from the altered function of one of the polypeptides that constitute the enzyme.

Morphological, clinical, biochemical and cell biological observations reported here provide evidence that ED type VII C resulting from the lack of activity of procollagen I N-proteinase really exists in human and is comparable to dermatosparaxis in calf and sheep. This autosomal recessive defect attracted the attention of animal geneticists because of a significant number of diseased animals resulting from inbreeding or artificial insemination. The incidence of the mutation, however, is probably low as only few reports of foci of the disease exist, two in cattle<sup>11,23</sup>, one in sheep<sup>13</sup>, one in dogs<sup>24</sup>, and one in cats<sup>25</sup>. The same might apply to humans. One of the initial patients reported as presenting procollagen I N-proteinase deficiency<sup>9</sup> was subsequently re-evaluated and found to be ED VII B. The clinical signs presented by the other patients, hip dislocation and joint laxity, indicate they belong to the forms A or B. The patients reported by Smith *et al.*<sup>30</sup> and our's described here seem to be the only real ED VII C. ED VII C remained unproved in humans until now partly because of its rarity, and inappropriate technical approach. Most centers investigating patients presenting with heritable disorders of collagen, routinely perform analysis of collagen in fibroblast cultures which does not provide a clear indication of enzyme deficiency<sup>10</sup>. Specific investigations as described here and also reported by Smith *et al.*<sup>30</sup> are required to provide a clear definition of the defect.

#### Methodology

**Clinical evaluation.** The pregnancy terminated at 29 1/2 weeks due to early rupture of fetal membranes. At 3 1/2 months, fontanelles were widely opened. High forehead, shallow supra orbital ridge, low nasal bridge, epicanthic folds and a semicircular skin fold extending from the inner canthus onto the cheek, were clearly visible. The nose was wide with a short tip. The philtrum was poorly chiselled. The maxilla vault was slightly narrow and retrognathia was noted. The neck was short. The abdomen was prominent with a large umbilical hernia. Genitalia showed hypoplasia of labia minora. Upper limbs seemed short with squared hands. Narrow hyperconvex fingernails were observed. Toe-nails were hypoplastic. Bilateral overlapping of second toe over third, fourth toe over third was present. Osteopenia was detected on skeletal radiographs.

**Transmission electron-microscopy.** The skin sample obtained at surgery was fixed in 4% glutaraldehyde in phosphate buffer pH 7.4, then fixed in 1% osmium tetroxide for 45 min, dehydrated in graded ethanol, embedded in Epon and stained by lead citrate and uranyl acetate. Examination was performed using an Elmiskop 100 Siemens.

**Extraction of skin collagen and SDS-PAGE analysis.** Skin samples of the patient collected from the resected hernia or from normal individuals of similar age collected during surgical procedures were preserved in 70% ethanol. Subcutaneous fat was scraped off using a scalpel blade. Skin samples were crushed into powder after freezing in liquid nitrogen and lyophilized, further fat was removed with petroleum ether and lyophilized again. Similar amounts of defatted

skin powder were sequentially extracted for 24 h at 4 °C (shaken in 0.15M NaCl, 0.05M Tris-HCl, pH 7.2, 0.05 mM NEM, 0.1 mM PMSF) followed by extraction for 24h at 4 °C in 0.5M acetic acid. The extracted collagen was collected by precipitation at 30% ethanol and analysed by slab SDS-PAGE electrophoresis<sup>28</sup> (7.5% acrylamide) under reducing or non-reducing conditions and staining by Coomassie blue. Purified collagen from dermatosparactic (D) calf skin similarly extracted by 0.15M NaCl and 0.5M acetic acid was run in parallel.

**Enzymatic digestion of the collagen precursors.** Samples of 25 µg of 0.5M extracted collagen from dermatosparactic calf skin and from the skin of the patient were incubated with 4 and 40 units of purified

procollagen I N-proteinase in 0.05 M Na cacodylate pH 7.2, 0.2 M KCl, 2 mM CaCl<sub>2</sub>, 2.5 mM NEM, 5 mM PMSF for 18 h at 26 °C. In one sample, the enzyme activity was inhibited by adding 80 mM EDTA. Pepsin digestion was also performed by incubating samples with 2.5 µg of pepsin at pH 3.0 for 16 h at 4 °C. After incubation, the samples were brought to 50% ethanol, centrifuged and the pelleted collagen was analysed by SDS-PAGE as above. (1 unit of PCP is the amount of endopeptidase required to process 1 µg of pN-I collagen in 18 h at 26 °C).

**Fibroblasts cultures and labelling experiments.** The cell culture, labelling, purification of labelled procollagen and electrophoretic procedures were as described previously<sup>29</sup>.

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