Detection and characterisation of an overmodified type III collagen by analysis of non-cutaneous connective tissues in a patient with Ehlers-Danlos syndrome IV

L Nuytinck, P Narcisi, A Nicholls, J P Renard, F M Pope, A De Paepe

Abstract

The clinical and biochemical observations in a patient with a mild form of Ehlers-Danlos syndrome (EDS) type IV are described. The patient's skin fibroblasts produced markedly diminished amounts of type III collagen. SDS-polyacrylamide gel electrophoresis of collagens produced by cells obtained from other, non-cutaneous tissues showed two forms of collagen $\alpha 1(\text{III})$ chains, a normal and a slow migrating, mutant form. Further analysis confirmed that the type III collagen molecules containing mutant α chains which were overmodified had a lower thermal stability and were poorly secreted into the extracellular medium.

The protein defect was mapped by *in situ* cyanogen bromide digestion and was located in $\alpha 1(\text{III})$ CB9, the C-terminal peptide of the collagen triple helix.

This study shows that non-cutaneous connective tissues can be a useful source for the study of type III collagen defects in patients with EDS type IV.

Ehlers-Danlos syndrome (EDS) is a heterogeneous group of inherited connective tissue diseases, sharing hyperextensible skin, easy bruising, dystrophic scarring, joint hypermobility, and tissue fragility as principal features.¹

In type IV or 'ecchymotic' EDS catastrophic ruptures of arteries or hollow organs occur, for which surgical repair is difficult or impossible because of the extreme fragility of the tissue. These are often fatal in the second or third decade.^{2,3}

EDS IV is clinically heterogeneous. In its most severe form the skin is very thin with a prominent venous pattern and pronounced bruising and scarring. The face, hands, and feet have a prematurely aged appearance (acrogeria). Facial features include large, prominent eyes, sharp nose, and thin lips.^{3,4} However, many EDS IV patients have a milder phenotype and complications arise later giving a longer life expectancy. The tissues affected in EDS IV are rich in type III collagen. All evidence to date suggests that EDS type IV is caused by abnormalities of this protein.⁵⁻¹⁰ An increasing number of collagen type III defects are now being identified and characterised at the DNA level.¹¹⁻¹⁶

Type III collagen is an homotrimeric molecule containing three identical protein chains encoded by a single complex gene. The protein is synthesised as a precursor which undergoes a series of post-translational modifications. The most notable of these are hydroxylation of specific proline and lysine residues and glycosylation of some of the hydroxylysines thus formed. From the random association of three α chains into trimers, it follows that a mutation in one of the COL3A1 alleles will affect 7/8 of the completed type III collagen molecules.¹⁷

This paper reports clinical data and biochemical analysis of a patient with a mild form of EDS IV, using conventional skin fibroblasts and cells derived from non-cutaneous tissues such as artery, vein, and peritoneum. The patient is heterozygous for a structural mutation in the collagen helix close to the C-propeptide of the collagen type III molecule.

Case report

The proband is a 54 year old woman with a lifetime history of easy bruising and recurrent bleeding and haematomas. She had suffered from varicose veins on the lower legs and from attacks of superficial phlebitis for several years. On three occasions her right shoulder had dislocated spontaneously. She was the fourth of seven sibs. Family history was otherwise unremarkable.

Clinical examination showed a short woman, below the 3rd centile for height (149 cm). Her facial features included prominent eyes with blueish sclerae, a pinched nose, and hypoplastic earlobes strongly suggestive of EDS IV (fig 1). The skin was generally thin and showed a prominent venous network but was not hyperextensible. Several ecchymoses were visible on the legs and abdomen. The knees and shins showed atrophic scars and haemosiderin deposits at the sites of old haematomas. There was hyperextensibility of the large joints (especially the elbows and knees), but mobility of the small joints was within normal limits. The Beighton score was raised at 5/9. The heart was clinically normal.

The patient's most recent hospital admission, at the age of 54 years, followed an acute volvulus of the sigmoid colon which was successfully reduced endoscopically. Shortly afterwards she developed a perforated bowel. At laparotomy, a superficial perforation of the sigmoid colon was observed and a sigmoidectomy was performed. Biopsies from artery,

Centre for Medical Genetics, University of Gent, De Pintelaan 185, B-9000 Gent, Belgium.

L Nuytinck
J P Renard
A De Paepe

Dermatology Research Group, Clinical Research Centre, Harrow, Middlesex HA1 3UJ.
P Narcisi
A Nicholls
F M Pope

Correspondence to Dr De Paepe.

Received 23 August 1991.
Revised version accepted 7 November 1991.

Figure 1 Facies of the patient at the age of 20 and 54 years, showing the typical features of EDS IV: large prominent eyes, sharp, long nose, and thin lips.

vein, peritoneum, and skin were obtained and cell cultures were established from the different tissues.

Material and methods

HISTOLOGY

Samples for light microscopy were fixed in formaldehyde and stained with haematoxylin and eosin; those for electron microscopy were fixed with 4% glutaraldehyde, 0.1 mol/l cacodylate buffer, pH 7.4.

CELL CULTURE AND LABELLING

Cell cultures were established from explants of skin, artery, vein, and peritoneum. Control skin fibroblasts were obtained from a healthy adult. The cells were maintained in Optimem-1 (Gibco-BRL) supplemented with 2% Ultrosor G (Gibco-BRL) and 5% fetal calf serum under standard conditions. Cells from passage 3 were grown on 50 mm plastic dishes and supplemented with 25 µg/ml ascorbic acid 24 hours before labelling.

Confluent cells were labelled for 20 hours in BME medium (FLOW laboratories) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mmol/l Hepes, 50 µg/ml β-aminopropionitrile, 25 µg/ml ascorbic acid, 5% dialysed fetal calf serum, and 1 µCi/ml ¹⁴C-proline. In some experiments incubation was at temperatures below 37°C and in others 0.3 mmol/l αα'-dipyridyl was added to the labelling medium.

After labelling, the medium was removed and proteinase inhibitors were added (50 µg/ml phenylmethylsulphonylfluoride (PMSF), 50 µg/ml N-ethylmaleimide (NEM), and 10 mmol/l EDTA final concentration). The cells were washed several times with phosphate buffered saline (PBS) and trypsinised from the dish. The pelleted cells were lysed at 4°C for one hour with 0.5 mol/l acetic acid containing 0.5% Triton X-100.

After αα'-dipyridyl labelling, cells were lysed with PBS (phosphate buffered saline)

containing 0.5% Triton X-100 and proteinase inhibitors. The lysates were combined with the medium for analysis.

PROCOLLAGEN AND COLLAGEN ISOLATION

Medium proteins were precipitated with two volumes of absolute ethanol at 4°C overnight. The precipitates were washed several times with 66% ethanol, dried, and finally redissolved in 0.5 mol/l acetic acid.

For the analysis of procollagens, aliquots were lyophilised and redissolved in electrophoresis sample buffer containing 2 mol/l urea and 1% β-mercaptoethanol.

To convert procollagen to collagen the alcohol precipitates were digested with pepsin (50 µg/ml, Boehringer) for six hours at 15°C. The pepsin was inhibited with pepstatin (0.05 µg/ml, Boehringer), the sample lyophilised, and redissolved in electrophoresis buffer without β-mercaptoethanol.

GEL ELECTROPHORESIS

Samples were analysed by 5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with the Laemmli buffer system¹⁸ containing 2 mol/l urea. Type III collagen was analysed under non-reducing conditions (as disulphide bonded trimers) and after delayed reduction (as monomers).¹⁹ After electrophoresis, the gels were processed for fluorography²⁰ and exposed to Kodak X-OMAT S films.

For two dimensional mapping, α chains were first separated by delayed reduction on SDS gels, then gel lanes were cut out and treated with cyanogen bromide (CNBr) in 70% formic acid for three hours at room temperature. The gel slices were re-equilibrated in gel sample buffer, embedded in 5% stacking gel, and electrophoresed in the second dimension on a 10% SDS-polyacrylamide gel. Radioactively labelled peptides were visualised by fluorography.

IMMUNOPRECIPITATION OF TYPE III COLLAGEN AND INCUBATION WITH αα'-DIPYRIDYL

Procollagen type III molecules produced in the presence or absence of αα'-dipyridyl were isolated by immunoprecipitation. Alcohol precipitates or lyophilised pepsin treated products were redissolved in PBS containing 0.05% NP-40. Goat antihuman type III collagen antiserum²¹ was added and left overnight at 4°C. Then rabbit antisheep antiserum was added and incubated at 4°C overnight. The immunoprecipitate was collected by centrifugation, the pellet washed with PBS + NP-40 solution, and finally dissolved in 0.5 mol/l acetic acid. Aliquots of this solution were either lyophilised directly (procollagens) or treated with pepsin (collagens).

THERMAL STABILITY OF COLLAGEN

The thermal stability of pepsin treated collagens was determined using trypsin digestion as

Figure 2 Electron microscopic appearance of the collagen fibrils in the patient's skin. Some of the collagen fibrils show an irregular shape (arrows).

a measure of the entity of disruption of the triple helical domain.^{11,22} Collagens were dissolved in 0.1 mol/l Tris-HCl, pH 7.5, 0.4 mol/l NaCl overnight at 4°C.

The temperature of the collagen solution was increased linearly from 33°C to 44°C at a rate of 15°C/hour. At temperature intervals of 1°C, aliquots were removed and digested with 100 µg/ml trypsin for two minutes at 20°C then 100 µg/ml ovomucoid trypsin inhibitor was added.

The reaction products were analysed by SDS-polyacrylamide gel electrophoresis with delayed reduction.

Figure 3 SDS polyacrylamide gel electrophoresis of collagens and procollagens from the EDS IV cells (P=peritoneum, V=vein, S=skin, A=artery) and from a control cell line (C=skin control). (A) Separation of the pepsin treated unreduced collagens. The $\alpha_1(\text{III})_3$ band showed marked diminution, especially in the skin fibroblasts of the EDS IV patient. There was a variable but higher expression of the $\alpha_1(\text{III})_3$ chains in the non-cutaneous tissues. (B) After delayed reduction ($\alpha_1(\text{III})$ chains running as monomers), all the cells from the different tissues of the patient showed a doublet in the position of reduced $\alpha_1(\text{III})$ chains with one component ($\alpha'_1(\text{III})$) migrating slower than the normal component. In the skin fibroblast the $\alpha'_1(\text{III})$ chains were also faintly visible. (C) Separation of the procollagens secreted into the medium by the patient's cells showed a doublet for the pro $\alpha_1(\text{III})$ chains whereas procollagens from the control showed a normal pattern.

RNA ISOLATION

Total cytoplasmic RNA was isolated from skin fibroblasts by the NP-40 lysis technique,²³ slot blotted onto nitrocellulose filters, and hybridised to nick translated cDNA probes specific for $\alpha_1(\text{III})$ and $\alpha_2(\text{I})$ mRNA. Quantitative estimates of type III mRNA levels were obtained by comparing signal intensity of the two probes.

Results

HISTOLOGY

Various tissues from the patient were examined by light and electron microscopy. In the skin, connective tissue fibres were sparse and collagen fibrils were irregular with marked variability in the diameter (fig 2). Some fibroblasts showed a dilatation of the rough endoplasmic reticulum.

In the distended bowel, the mucous membrane and the muscularis mucosae were relatively well preserved. The submucosa was generally thin with few fibroblasts and irregular, fragmented collagen fibres.

In several areas the deep muscle layer showed inflammatory infiltration and intramural bleeding. In other sites the muscularis was narrow and disrupted by distension. There was serositis and mesothelial proliferation. There were striking vascular malformations and telangiectasias.

ANALYSIS OF PROCOLLAGENS AND COLLAGENS

Analysis of unreduced pepsin treated collagens secreted into the medium by the patient's skin fibroblasts showed barely visible amounts of type III collagen. In contrast, cells from the

other tissues (peritoneum, vein, and artery) showed higher but variable amounts of type III collagen (fig 3A). After delayed reduction a doublet consisting of normal and slow migrating α_1' (III) chains was clearly visible in the medium of cells from non-cutaneous tissues and faintly detectable in that from the skin fibroblasts (fig 3B). Examination of the cellular proteins showed that a significant amount of the slow migrating component was retained intracellularly (data not shown). Two forms of type III pro α chains were also observed after separation of procollagens under reducing conditions (fig 3C).

To prove the slow migrating α chains were from type III collagen, the pepsinised collagens were separated unreduced on a first dimension gel and in the second dimension after in situ reduction with β -mercaptoethanol. The patient's pattern showed two bands corresponding to type III collagen, compared to the expected single spot from the control cell line (fig 4A). When the cells were incubated with $\alpha\alpha'$ -dipyridyl and the type III procollagens immunoprecipitated, electrophoresis showed only a single band for type III collagen (fig 4B). A similar result was obtained when $\alpha\alpha'$ -dipyridyl treated samples were pepsinised and treated in the same way (data not shown). When cells were incubated at 30°C the amount of type III collagen secreted into the medium was increased (data not shown).

MELTING CURVE

Exposure of collagen molecules to increasing temperatures and digestion with trypsin showed that molecules containing the mutant α_1' (III) chains were degraded at 38°C, 2°C lower than the normal type III collagen molecules from the patient and from a normal control (fig 5).

CYANOGEN BROMIDE (CNBr) DIGESTION OF THE TWO COLLAGEN TYPE III COMPONENTS

Two dimensional cyanogen bromide mapping of the unreduced type III collagen from the patient showed quite a normal peptide pattern although there were some differences, especially in the α_1 (III) CB5 (fig 6). Mapping of the type III collagen components, separated by electrophoresis with delayed reduction, showed a triplet of spots in the CB5, CB9 region. The patient's normal α_1 (III) chain gave a single spot for both peptides comparable to control sample whereas the slow migrating α_1' (III) chains had a doublet of spots for these peptides, both migrating slower than the normal peptides. The partial cleavage products higher up the gel were also tilted, showing the presence of both normal and slow migrating components.

RNA ISOLATION

Steady state levels of mRNA for type III collagen were normal (data not shown). This indicates that the reduction of type III collagen in the medium was not because of

Figure 4 (A) Two dimensional separation of α_1 (III) chains to verify the origin of the doublet which was visible only after separation of the collagen molecules with delayed reduction. In the first dimension the pepsin treated molecules were separated unreduced, reduced in vitro, and separated in the second dimension. The patient's type III collagen shows a clear doublet in the second dimension whereas the control gives a single spot. EDS IV patient: V = vein, P = peritoneum, S = skin, A = artery. C = skin control. (B) Separation on SDS-PAGE of the immunoprecipitated procollagens produced by the arterial fibroblasts in the presence of $\alpha\alpha'$ -dipyridyl. Lanes 1 and 7, control medium (without $\alpha\alpha'$ -dipyridyl), lane 2, EDS IV medium + cells (without $\alpha\alpha'$ -dipyridyl), lanes 3–6, EDS IV medium + cells incubated in the presence of $\alpha\alpha'$ -dipyridyl in different concentrations. Incubation of the cells with $\alpha\alpha'$ -dipyridyl abolished the doublet for the immunoprecipitated pro α_1 (III) showing that the slow migration was the result of excessive post-translational modification. – = incubation without $\alpha\alpha'$ -dipyridyl. + = incubation with $\alpha\alpha'$ -dipyridyl.

decreased synthesis, but the result of poor secretion and intracellular accumulation and degradation of the mutant collagen.

Discussion

This patient shows many of the clinical features of Ehlers-Danlos syndrome type IV,¹ but she is not typical. She is not acrogeric and

Figure 5 Thermal denaturation of collagens from the control cell line (A) and from the EDS IV patient (B). Normal type III collagen from the patient and from the control melted at 40°C; the slow migrating α_1' (III) chains melted at 38°C.

Figure 6 (A) Two dimensional mapping of CNBr peptides of the collagen chains synthesised by cells of the patient. The CB peptide pattern obtained from the unreduced α_1 (III)₃ chains is indistinguishable from that of the control cell lines. The CB peptide pattern of the reduced α_1 (III) chains showed a triplet of spots in the CB peptides α_1 (III) CB5 and α_1 (III) CB9 (arrow) and a marked distortion of the spots representing partial cleavage products higher up the gel. (B) Linear map of CB peptides of the α_1 (III) chain.

has survived until her mid fifties before suffering serious vascular or intestinal problems, while patients with classical EDS IV die of vascular ruptures at a relatively early age. She must, therefore, be considered to have a mild variant of the disorder.

Nevertheless, the initial collagen analysis of skin fibroblast cultures from this patient showed the marked deficiency of type III collagen characteristic of EDS IV patients. It was the opportunity to investigate cells from non-cutaneous tissues, which apparently produce more type III collagen than skin fibroblasts, that first indicated the presence of the slow migrating α_1 (III) chains. These were subsequently observed in skin fibroblasts when techniques were developed to concentrate and purify the small amounts of type III collagen produced by these cells.

Although some mutant α_1 (III) chains were found in the culture medium of cells from all tissue sources, analysis of intracellular proteins showed that mutant molecules were preferentially retained within the cells, presumably destined for degradation. By reducing the incubation temperature of the cells the secretion of type III collagen could be improved considerably. A similar observation has been made with cells from some other patients with EDS IV.²⁴ This may reflect the thermal instability of the mutant molecules which have a melting temperature some 2°C lower than normal. It may also explain why cooler superficial tissues, such as skin, are less dramatically affected than internal organs.

Slow migration of collagen α chains may result from an insertion of peptide sequences or from excessive post-translational modification of lysine residues. To control the nature of the slow migration, cells were incubated with $\alpha\alpha'$ -dipyridyl, an iron chelator that inhibits the enzymes responsible for post-translational hydroxylation. It will thus abolish any changes owing to variation in the post-translational modification and distinguish them from peptide insertion. In this patient the presence of a single band for type III collagen and procollagen after treatment with $\alpha\alpha'$ -dipyridyl showed that the slow migration of the α_1 (III) chains was the result of excessive post-translational modification.

In the normal collagen molecule, triple helix formation starts at the C-terminus and moves towards the N-terminus. If a mutation in the protein delays this process then sequences N-terminal of the mutation remain substrates for the modifying enzymes and become excessively modified.²⁵ A consequence of this is that locating the onset of overmodification within the molecule gives an approximate location of the mutation. This can be achieved by in situ cyanogen bromide digestion of gel separated normal and mutant α chains. In this patient the mutant type III collagen gave two spots for the peptides α_1 (III) CB5 and α_1 (III) CB9. Both migrated more slowly than the single spot obtained for these two peptides in the normal α_1 (III) chain and were thus overmodified (fig 6). Since α_1 (III) CB9 is the most C-terminal of the CB peptides (fig 6B) a mutation in this peptide was indicated.

These results were further substantiated by cleavage of the protein with a vertebrate-like collagenase which cleaves α_1 (III) CB9 from the rest of the molecule before cyanogen bromide digestion (Nicholls *et al*, in preparation). This

clearly showed an overmodified CB9 peptide. The location of the mutation has now been confirmed by PCR amplification, cloning, and sequencing of the cDNA encoding $\alpha 1(\text{III})$ CB9 and identification of a G to T substitution converting glycine residue 910 to valine.^{26,27}

So far very few type III collagen abnormalities have been published; these include four point mutations, Gly to Arg 619,¹⁵ Gly to Ser 790,¹² Gly to Asp 883,¹³ and this Gly to Val 910. The patients with Gly to Ser 790 and Gly to Asp 883 produce normal and slow migrating $\alpha 1(\text{III})$ chains, (P Narcisi, personal communication) while the patient with Gly to Arg 619 produces a reduced amount of electrophoretically normal $\alpha 1(\text{III})$ chains. The clinical phenotype of these point mutations is quite variable: Gly to Ser 790 is associated with severe EDS IV, both Gly to Asp 883 and this patient have relatively mild disease while the Gly to Arg 619 was associated with familial aortic aneurysms. This variability must reflect subtle differences in the effects of the mutations on the physical and biological properties of the resultant proteins. Many more characterisations of type III collagen mutations will be required before any correlation between mutation and clinical phenotype can be meaningful. By analogy to type I collagen, both the position and nature of the mutations are factors²⁸ in determining disease severity.

This study has also shown the utility of culturing cells from non-cutaneous, type III collagen rich tissues wherever possible. Their greater production of type III collagen facilitates the identification of mutations in this protein compared to the more routinely available skin fibroblasts. However, the latter can be used if steps are taken to concentrate and purify the type III collagen before analysis.

We are indebted to Dr J De Roose, thoracic-vascular surgeon, who provided us with biopsies from the patient, and to Drs G Quatacker and A Dierick from the N Goormaghtigh Institute for Pathological Anatomy, who performed the morphological and ultrastructural studies on this patient. This work was supported in part by the EC Concerted Action on Heritable Connective Tissue Disorders 1990–1992, project leader Dr M Matton. Part of the collagen analysis was carried out while L Nuytinck was a visiting worker at the MRC Dermatology Research Group at the Clinical Research Centre, Harrow.

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