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An elastin gene mutation producing abnormal tropoelastin and abnormal elastic fibres in a patient with autosomal dominant cutis laxa

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Elastin is the protein responsible for the characteristic elastic properties of many tissues including the skin, lungs and large blood vessels. Loss-of-function mutations in the elastin gene are known to cause the heart defect supravalvular aortic stenosis (SVAS). We and others have identified deletions, nonsense mutations and splice site mutations in SVAS patients that abolish the function of one elastin gene. We have now identified an elastin mutation in a patient with a completely different phenotype, the rare autosomal dominant condition cutis laxa. A frameshift mutation in exon 32 of the elastin gene is predicted to replace 37 amino acids at the C-terminus of elastin by a novel sequence of 62 amino acids. mRNA and immunoprecipitation studies show that the mutant allele is expressed. Electron microscopy of skin sections shows abnormal branching and fragmentation in the amorphous elastin component, and immunocytochemistry shows reduced elastin deposition in the elastic fibres and fewer microfibrils in the dermis. These findings suggest that the mutant tropoelastin protein is synthesized, secreted and incorporated into the elastic matrix, where it alters the architecture of elastic fibres. Interference with cross-linking would reduce elastic recoil in affected tissues and explain the cutis laxa phenotype.

INTRODUCTION

Elastin is the protein responsible for the characteristic elastic properties of many tissues. The elasticity of skin, lungs and large blood vessels depends on elastic fibres in the extracellular matrix. These are composed of an amorphous and a microfibrillar component; the amorphous component, comprising ~90% of the mature elastic fibre, is made of elastin. The 45 kb human elastin gene maps to 7q11.2 and comprises 34 exons (1–4). Elastin initially is synthesized as tropoelastin, a soluble polypeptide of ~72 kDa, with a characteristic primary structure of alternating hydrophobic and cross-linking domains. After secretion, the individual tropoelastin molecules are aligned on a scaffold of fibrillin-rich microfibrils. This alignment is stabilized by the formation of intermolecular cross-links (desmosines). Oxidative deamination and cross-linking of certain lysine residues catalysed by the enzyme lysyl oxidase produce a highly insoluble network of elastic fibres. In this network, the hydrophobic domains are thought to form a loose and very mobile structure of stacked β-sheets with β-turns that is responsible for the resilience of the protein.

Elastin is a major component of the aorta and large arteries. Supravalvular aortic stenosis (SVAS), a congenital narrowing of the ascending aorta (5), is caused by haploinsufficiency for elastin as a result of disruption (6,7), intragenic deletions (8,9) or point mutations (3,4) in the elastin gene. SVAS also occurs as part of Williams syndrome (WS; MIM 194050), where a microdeletion at 7q11.23 results in hemizygosity at a series of contiguous gene loci including the elastin locus (10). Elastin is also a major component of skin and, in an attempt to identify other categories of elastin mutation that might cause different connective tissue disorders, we have studied a family with autosomal dominant cutis laxa.

Cutis laxa (CL) comprises a rare and heterogeneous group of disorders characterized by lax inelastic skin with or without internal manifestations (11). The skin of CL patients tends to hang in loose folds, giving an appearance of premature ageing. In contrast to Ehlers–Danlos syndrome, the skin in CL is neither hyperelastic nor fragile; wound healing is normal and there is no joint hypermobility (12). Congenital CL may follow autosomal dominant, autosomal recessive or X-linked inheritance (13). Autosomal recessive CL may present at birth or may develop later during infancy, and CL may be acquired following trauma or febrile exanthema. Pulmonary and cardiovascular involvement have also been described in autosomal recessive CL, where complications often lead to death during childhood (14,15). Autosomal dominant CL is associated with a more normal lifespan and internal abnormalities are less frequent. Among those reported are pulmonary artery stenosis (14,16,17), angiographic

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abnormalities of the aorta and great vessels (18), emphysema (19), bronchiectasis (13), hernias and genital prolapse (20).

Deficiency of lysyl oxidase has been reported to cause X-linked recessive CL (21), but the molecular pathology of the other forms has yet to be defined. In this study, we have identified a mutation in the elastin gene that causes autosomal dominant CL. Abnormal elastin protein is produced which, if associated with the microfibrils, would be predicted to result in a looser network of elastic fibres with less elastic recoil.

RESULTS

Clinical diagnosis

The proband (AS) is a 37-year-old Caucasian woman. As a baby she was noted to have heavy folds of fat under her chin and across her chest, abdomen, groins and thighs (Fig. 1). Her first facial cosmetic surgery was at 4 years of age, with six subsequent operations. Bilateral herniorraphies were performed at 7 years of age. A cardiac murmur was noted at 4 years of age, and when she presented with reduced exercise tolerance at 19 years, right ventricular hypertrophy was noted. Right ventricular angiography showed hypertrophy of the infundibulum with angiographic narrowing. There were multiple peripheral pulmonary stenoses including stenoses in very small vessels. She has no skin hyperelasticity, and wound healing has always been good. Since late adolescence, she has suffered from Raynaud's phenomenon and is not taking medication for her condition. The condition in this family is autosomal dominant (Fig. 3a). The father (described in ref. 12) had loose skin on his chest, forearms and abdomen in addition to facial drooping, and had two cosmetic operations in his teenage years. He had required an inguinal herniorraphy, but apart from a venous thrombosis at the age of 50 years, has remained in good health.

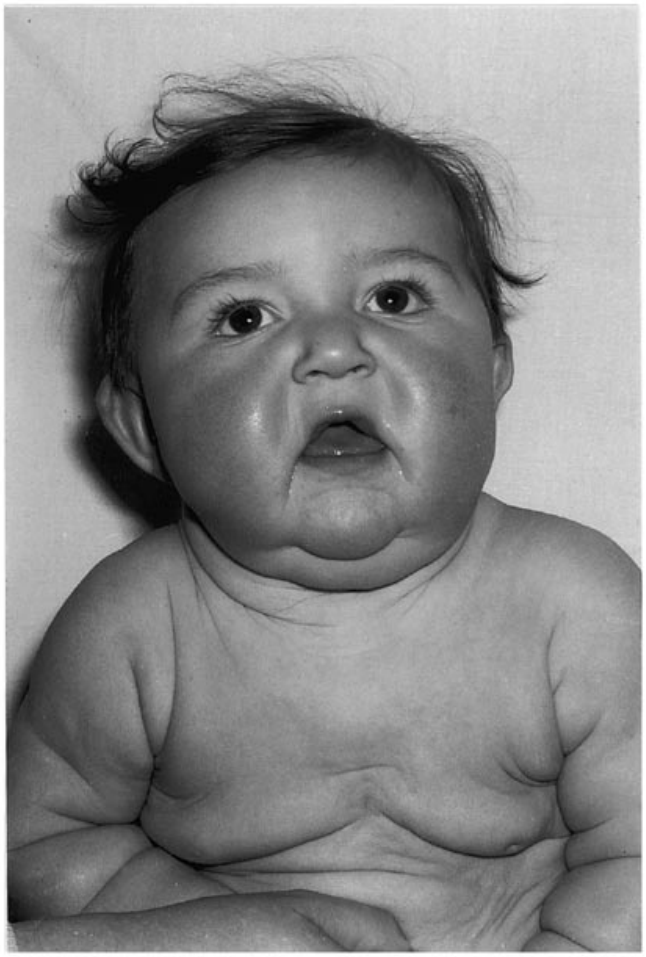

Figure 1. Patient AS with autosomal dominant cutis laxa; age 10 months, showing redundant folds of skin on face, arms and trunk.

Histological and immunocytochemical features in CL skin

Elastin was present, in normal skin, as fine subepidermal elastin arcades with a deep reticular layer of thicker, mainly parallel, fibres. In AS, the quantity and architecture of elastin were grossly abnormal. There was a complete absence of subepidermal elastin fibres. In addition, markedly reduced quantities of reticular elastin were observed, and the fibres appeared fragmented (Fig. 2c and d).

Tropoelastin and elastin immunostaining of control and patient skin sections exactly paralleled the histological findings (Fig. 2a and b). In normal skin, fibrillin was observed as tiny, fine fibres perpendicular to the dermo-epidermal junction and as scattered fibre bundles in the reticular dermis. In contrast, fibrillin was absent from the subepidermal area in the skin of AS, with small fragmented fibres present in the reticular dermis and perivascular staining in the dermis (data not shown).

Electron microscopy of CL skin

At the ultrastructural level, oxytalan fibres appeared normal and were detected at the dermo-epidermal junction, streaming into the papillary dermis. Here, some fibres showed a vacuolated, 'moth-eaten' appearance (Fig. 2e and f). In the reticular dermis, many elastin fibres were seen to be highly abnormal in both shape and electron density (Fig. 2g and h). Situated within a loose matrix of widely dispersed collagen bundles, they showed extensive fragmentation and branching of the amorphous component. A particularly striking feature was the increasing electron density from the inner to the outer regions of complex branching fibres, as well as the homogeneous nature of the amorphous elastin in the branches compared with the main fibre. There was no apparent excess of the microfibrillar elements of elastin in this specimen.

Elastin gene mutation analysis

There was no evidence of a large deletion in patient AS: fluorescence *in situ* hybridization (FISH) analysis using an elastin cosmid probe showed signals on both copies of chromosome 7, and she was heterozygous for a polymorphic microsatellite in intron 18 of the elastin gene (data not shown). PCR products containing each exon of the elastin gene were amplified from genomic DNA of AS and screened by combined single strand conformation polymorphism (SSCP) and heteroduplex analysis (3). Exon 32 gave an abnormal band, not seen in 60 controls, and sequencing revealed deletion of a single adenine in codon 748 (Fig. 3b). The mutation was confirmed by sequencing cloned PCR products and by using an allele-specific amplification refractory mutation system (ARMS) primer to amplify the mutant allele selectively (Fig. 3c). The mutation was also present in the proband's father (RW) but not her mother (EW) or her unaffected

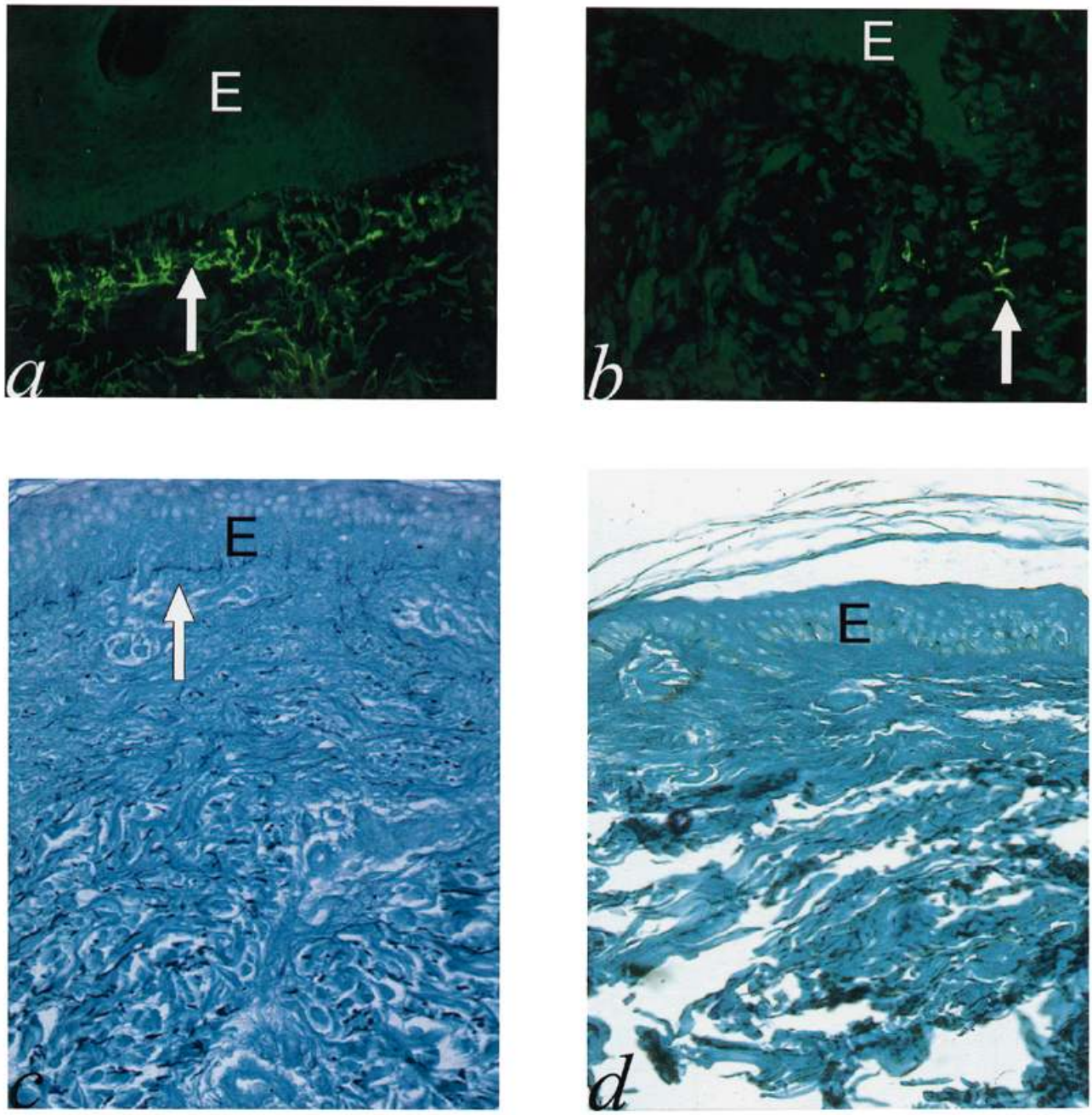

Figure 2. Comparison of normal and CL skin. (**a** and **b**) Tropoelastin immunocytochemistry. Spatial pattern in normal skin (a), parallels that in (c); in the patient, tropoelastin is markedly reduced in the sub-epidermal region with reduced and fragmented deep fibres (b). (**c** and **d**) Aldehyde fuchsin staining. Note the sub-epidermal arcades of elastin and deep reticular fibres in control (c) and absence of sub-epidermal arcades and fragmentation of sparse deep fibres in patient (d). (Magnification ×25; E = epidermis). *Continued overleaf.*

daughter (CS). It is predicted to cause translation to proceed 76 bases into the 3′-untranslated region before encountering a stop codon, replacing the C-terminal 37 amino acids of the normal elastin with a sequence of 62 novel amino acids (Fig. 4).

Elastin gene expression

mRNA was isolated from cultured skin fibroblasts of AS and two age-matched controls. Quantitative dot-blot analysis showed that the ratio of elastin to β-actin mRNA was similar in AS and the controls (Table 1), suggesting that the mutant mRNA is as stable as the normal form. The presence of the mutant mRNA was confirmed by ARMS using the exon 32 mutant ARMS forward primer and a reverse primer designed from the cDNA sequence of exon 33. The mutant allele could be amplified selectively from cDNA of the patient AS but not from two control samples (Fig. 3d).

Exon 32 has been reported to be alternatively spliced out (22). Amplifying across exons 30–33 revealed two isoforms in both patient and control cDNA samples (Fig. 3e). Sequencing gel-purified samples confirmed that the 195 bp product contained

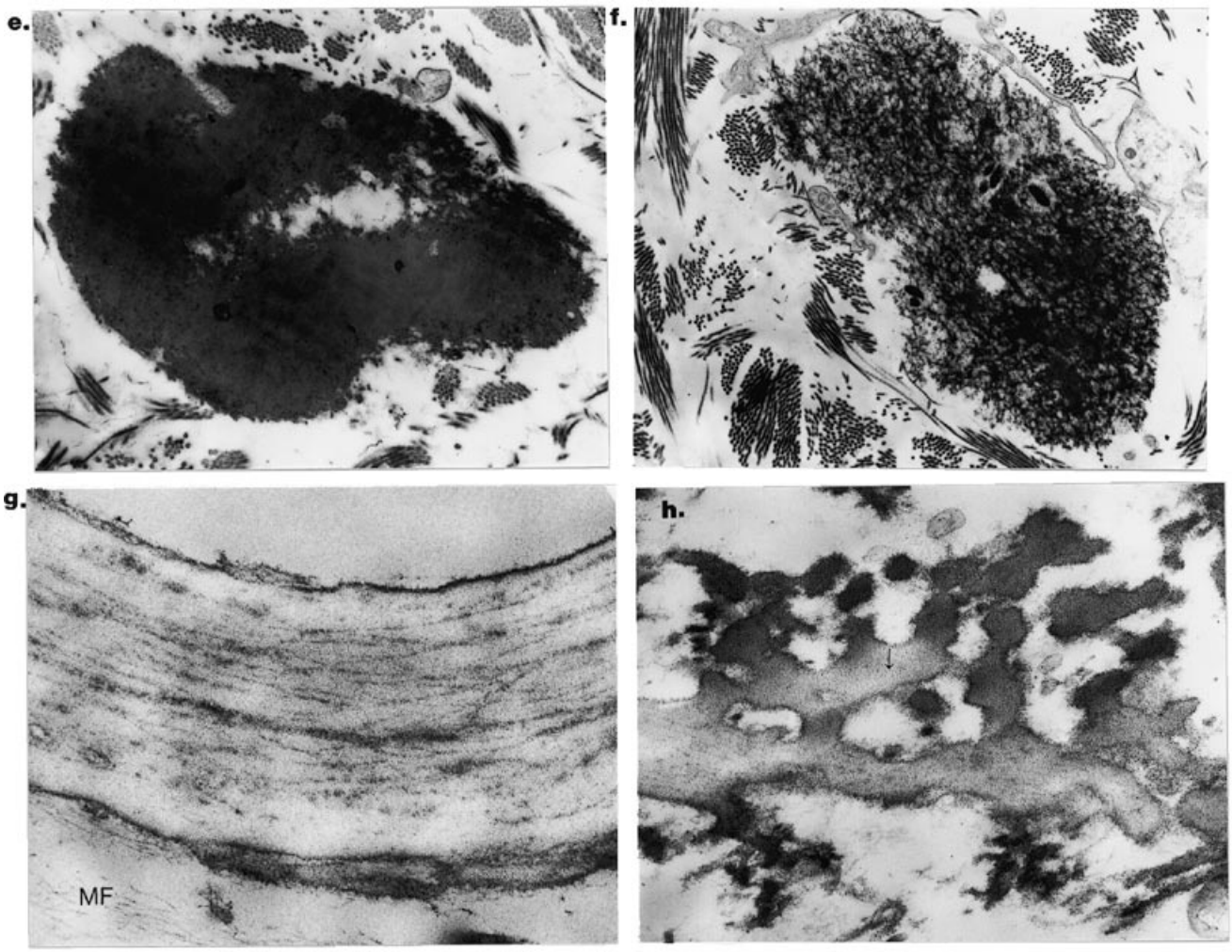

Figure 2. Comparison of normal and CL skin. (**e** and **f**) Electron micrographs (×14 400) of elastin fibre in the papillary dermis. Note the 'moth-eaten' appearance in the patient (f) compared with the uniform density in normal skin (e). (**g** and **h**) Electron micrographs (×56 000) of elastin from the reticular dermis. Note the uniformly electron-lucent core, longitudinal electron-dense inclusions and absence of branching in normal skin (g). A few microfibrils (MF) can be seen. In the patient (h), there is abnormally branched elastin, an increase in electron density in the peripheral branches, and a degree of internal homogeneity (arrow) compared with the pale, central part which contains electron-dense inclusions.

exon 32 and the 141 bp product lacked this exon (data not shown). The 195 bp band from the patient contained a mixed population of mutant and normal exon 32 alleles, confirming the ARMS results.

Tropoelastin synthesis and secretion

Dermal fibroblasts from AS and a control individual were cultured in the presence of [^{14}C]amino acids. The expressed tropoelastin was immunoprecipitated from medium, and sequentially from detergent-soluble and guanidine HCl-soluble cell layers. Immunoprecipitates were resolved on 8% SDS–PAGE gels and visualized by fluorography. Compared with the control, the patient's tropoelastin showed two distinct additional bands with slower electrophoretic mobility (Fig. 5). These isoforms are probably larger in size than normal alternatively spliced isoforms, and were seen in both the medium and detergent-soluble cell layer of AS. These results indicate that mutant protein is synthesized and secreted. No tropoelastin was demonstrated in the guanidine-extracted cell layers of either AS or controls.

Table 1. Quantitation of elastin expression by slot-blot analysis

Gross c.p.m.	AS mRNA (4 μg)	Control 1 mRNA (4 μg)	Control 2 mRNA (4 μg)	AS mRNA (0.5 μg)	Control 1 mRNA (0.5 μg)	Control 2 mRNA (0.5 μg)
Elastin	4689	4927	4657	489	544	500
β-Actin	3821	4181	3838	607	593	576
Elastin/ β-actin ratio	1.22	1.18	1.21	0.80	0.91	0.87

The amount of elastin expressed is calculated relative to β-actin. Levels of elastin mRNA are similar in patient (AS) and control fibroblasts.

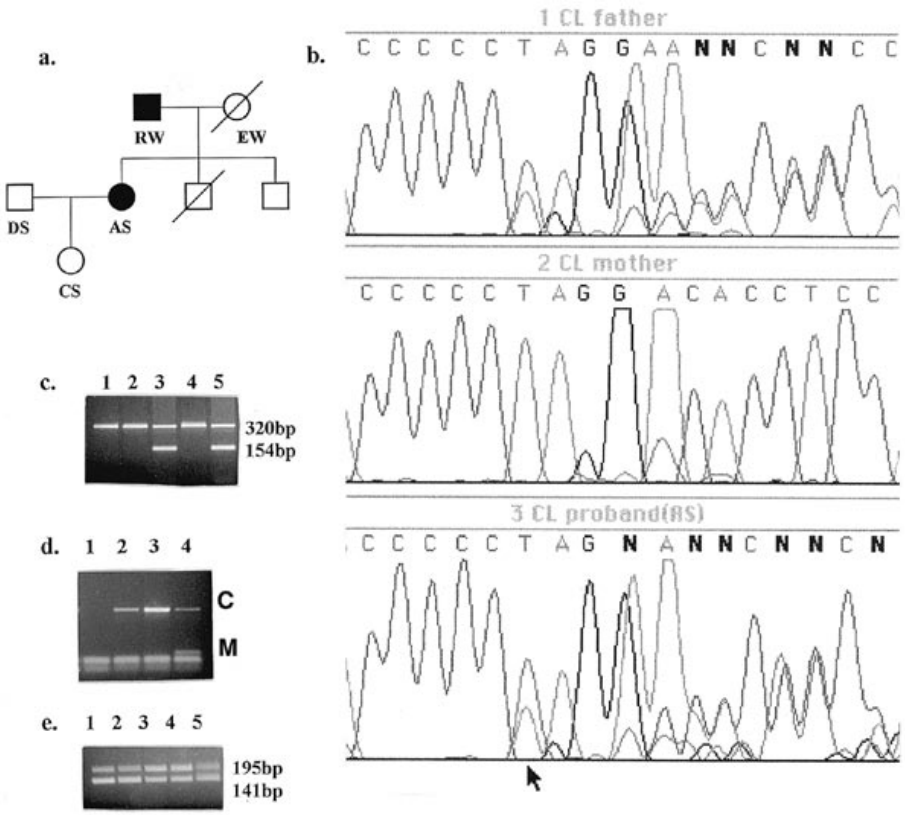

Figure 3. Molecular analysis. (**a**) Cutis laxa family pedigree. (**b**) Antisense double strand sequencing shows the proband (AS) and her father (RW) are heterozygous for a single base (T) deletion. (**c**) Confirmation of the mutation by ARMS PCR analysis. The ARMS primer selectively amplifies the mutant allele (154 bp) in AS and RW. Internal control (320 bp). Lane 1, control; lane 2, EW; lane 3, AS; lane 4, CS; lane 5, RW. (**d**) Mutation detection in patient cDNA. The ARMS primer selectively amplifies the mutant allele (M = 110 bp) only in AS. Internal human β-actin control (C = 330 bp). Lane 1, blank; lanes 2 and 3, controls; lane 4, AS. (**e**) RT-PCR showing the alternatively spliced isoforms of exons 30–33 of the elastin cDNA. The 195 bp product comprises exons 30, 31, 32 and 33; the 141 bp product comprises exons 30, 31 and 33. Exon 32 is spliced out.

Structural analysis of the predicted modified protein

PROSITE (23) and PRINTS (24) searches showed no matches of the predicted novel amino acid sequence to other genes or motifs. The sequence is quite hydrophilic. Structural predictions were made using the Protein Analysis server (http://bmerc-www.bu.edu/psa/) with a type 2 analysis for a protein fragment. This gave strong loop predictions throughout, plus helical propensity between residues 756 and 767, mainly based on amphipathy. There was no significant strand or turn prediction, so this segment may form a short transient helix periodically. Further analysis using the latest version of the protein secondary structure prediction program, GORBTURN (25,26) showed strong turn/loop propensities predicted throughout the sequence, and some propensity for helix formation between residues 753 and 764 of the altered sequence. Overall, the analysis indicates an easily hydrated and probably flexible and dynamic structure.

DISCUSSION

Haploinsufficiency for elastin often causes SVAS, but not CL. Patients with Williams syndrome, who have large deletions encompassing the whole of the elastin gene (10), frequently suffer from SVAS, as do patients heterozygous for disruptions, deletions or point mutations in the elastin gene (3,4,6–9 and our unpublished data). In contrast with these loss-of-function mutations, the mutation in our patient and her father is predicted to produce a stable but abnormal tropoelastin.

Elastic fibres in the skin of our patient were both deficient in quantity and abnormal in structure. The elastin fibrils were vacuolated, fragmented and branched, and fibres of the reticular dermis showed excessive variation of electron density. Reports of skin histology in other patients with CL (not necessarily the dominant form we describe) have been generally similar (27–29); at the ultrastructural level, a wide range of features have been reported in both congenital and acquired CL, including the presence of excess microfilaments, a paucity of the amorphous component, globular fibrils and 'moth-eaten' elastin (27–29).

Elastic fibres are complex structures of the extracellular matrix that do not assemble spontaneously. Microfibrils are believed to direct the alignment and polymerization of tropoelastin, which binds specifically to the acidic domain of microfibril-associated glycoprotein (MAGP) (30). Assembly of the elastic fibre requires precise non-covalent packing of tropoelastin to the growing elastin matrix to provide the alignment of cross-linking sites within each monomer.

These observations favour the hypothesis that the exon 32 frameshift mutation led to the production of abnormal elastic fibres in our CL patient. We have shown that the levels of elastin mRNA are comparable with normal controls and that the mutant transcript is produced, is stable [probably because it retains the

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      ... Exon 32                          ↓                                                  Exon 33
741    A   A   G   L   G   G   V   L   G   G   A   G   Q   F   P   L   G  |G   V   A
N     gct gct ggc ctt gga ggt gtc cta ggg ggt gcc ggg cag ttc cca ctt gga gga gtg gca
AS    gct gct ggc ctt gga ggt gtc ctg ggg gtg ccg ggc agt tcc cac ttg gag gag tgg cag
                                  L   G   V   P   G   S   S   H   L   E   E   W   Q

                                                          Exon 34 (= bovine exon 36)
761    A   R   P   G   F   G   L   S   P   I   F   P  |G   G   A   C   L   G   K   A
N     gca aga cct ggc ttc gga ttg tct ccc att ttc cca ggt ggg gcc tgc ctg ggg aaa gct
AS    caa gac ctg gct tcg gat tgt ctc cca ttt tcc cag gtg ggg cct gcc tgg gga aag ctt
       Q   D   L   A   S   D   C   L   P   F   S   Q   V   G   P   A   W   G   K   L

781    C   G   R   K   R   K  STOP
N     tgt ggc cgg aag aga aaa tga
AS    gtg gcc gga aga gaa aat gag ctt cct agg acc cct gac tca cga cct cat caa cgt tgg
       V   A   G   R   E   N   E   L   P   R   T   P   D   S   R   P   H   Q   R   W

AS    tgc tac tgc ttg gtg gag aat gta aac cct ttg taa
801    C   Y   C   L   V   E   N   V   N   P   L  STOP
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Figure 4. Predicted effect of the mutation. Deletion of an adenine in codon 748 in exon 32 (↓) causes a frameshift that replaces the 37 C-terminal amino acids of elastin with a novel sequence of 62 amino acids. In some isoforms, the frame-neutral exon 32 is spliced out, and the mutant and normal alleles will produce the same product when this happens. N, normal amino acid sequence; AS, sequence in cutis laxa patient. Exon boundaries are marked (|). Codons are numbered as in ref. 3.

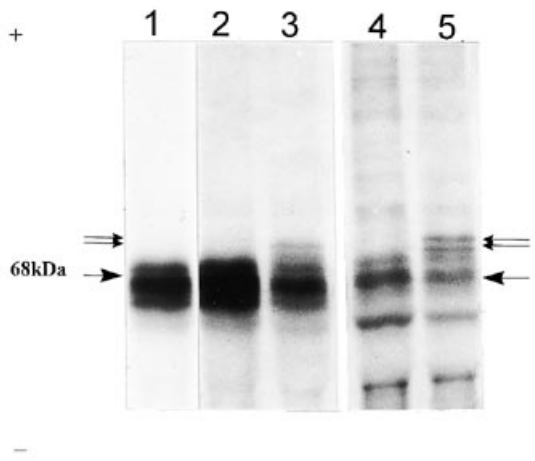

Figure 5. Immunoprecipitation of newly synthesized tropoelastin. Fluorographs of tropoelastin immunoprecipitates from medium and detergent-soluble cell layer fractions of patient (AS) and control fibroblasts. In addition to the tropoelastin bands in the controls, patient fibroblasts synthesized two higher M_r tropoelastin forms. The large arrow highlights the major tropoelastin isoform in the controls. The small arrows indicate the two higher M_r bands present only in AS. Lanes 1, 2 and 4, control fibroblasts (lanes 1 and 2 are the same track exposed in 1 to show the isoform pattern, and in 2 to show the high M_r patient bands); lanes 3 and 5, patient fibroblasts; lanes 1–3, medium immunoprecipitates; lanes 4 and 5, cell layer NET extract immunoprecipitates.

normal 3′-untranslated region (22)] and is translated. We propose that the mutant tropoelastin is incorporated into the elastin matrix, but then packs abnormally. All the major identified sites of cross-linking (31) are present (between domains in exons 10, 19 and 25), but the C-terminal structure may prevent them aligning correctly. Tropoelastin binds MAGP mainly through a disulfide-linked loop in the C-terminal part of the tropoelastin molecule and the four C-terminal residues which are basic (30); in the mutant, these are replaced by a highly hydrated and flexible tail with little secondary structure and lacking MAGP-binding sites. Abnormal packing could lead either to cessation of growth of the elastin matrix from the microfibril or to abnormal fibres containing incorrect cross-links. The abnormal elastin fibres would result in a loose framework with less elastic recoil, explaining the lax skin, hernias and peripheral pulmonary stenoses in our CL patient. They may also be more susceptible to degradation by metallo-proteases or elastase, which could explain our observation of less elastin deposition in the fibres, fewer microfibrils and vacuolated, 'moth-eaten' structures.

The relatively mild CL phenotype of our patient may stem from the alternative splicing that allows the mutant allele to produce a proportion of a normal elastin isoform by skipping exon 32. Since peripheral pulmonary stenoses have been described in other CL patients (14,15), it seems likely that the association is also based on an underlying elastin defect. Further studies into the molecular pathology of this type of CL may be important for the treatment and management of the disorder. The possibility of using elastase inhibitors to prevent vascular artery stenoses has been suggested (32). Pre-natal diagnosis can also be offered to families with dominant cutis laxa, which will allow early diagnosis and treatment.

MATERIALS AND METHODS

Histology

One full-thickness (4 mm) punch biopsy was taken from the forearm of the patient and from a healthy 32-year-old female volunteer on no medication and processed for histology as described previously (33).

Immunostaining

Immunostaining was carried out using fibrillin 5507 and tropoelastin antibodies using the methods described previously (33). The elastin antibody was raised against α-elastin (i.e.

extracted elastin as in tissue elastic fibres); the fibrillin antibody was raised against intact microfibrils, and recognizes fibrillin-1 and fibrillin-2 (33).

Electron microscopy

A 4 mm punch biopsy of skin was removed from the inner arm of the patient, and processed for electron microscopy as previously described (34). Age- and sex-matched skin obtained from the inner arm of a control individual was processed similarly.

Mutation detection and sequencing

Exon 32 of the *ELN* gene was PCR amplified using the conditions and primers described previously (3). Mutations were detected by a combination of SSCP and heteroduplex analysis (3). Mutations were characterized initially by direct double-strand fluorescent dye terminator cycle sequencing (ABI Prism) of column-purified PCR products in both orientations with a matched control on a fluorescent sequencer (ABI 373).

To confirm the mutation, exon 32 PCR products were cloned into PCR2.1 (Invitrogen) and sequenced in both orientations using both exon 32 PCR primers. The mutation was confirmed in at least four individual clones.

Confirmation of mutation by ARMS

ARMS PCR was carried out using the elastin exon 32R primer and a forward primer designed to amplify the mutant allele selectively (154 bp). *ELN* X32R: 5′ CCTTGTGTGGA-CATGGGCTCTGG; *ELN* X32F ARMSM: 5′ CTG CTG GCC TTG GAG GTG TCG TG. An internal control was included, which gave a 320 bp product. Standard PCR conditions were used (3). Products were separated on a 2% agarose/TBE gel.

RT-PCR analysis

Skin fibroblast cell cultures were established and maintained using standard tissue culture protocols. Cultures were used at passages 4–7 and total RNA was isolated using the RNeasy kit (Qiagen). Total RNA (1 μg) from each sample was reverse transcribed using a first-strand cDNA synthesis kit (Promega). The cDNA (50 ng) was amplified using primers from within exons 30–33 of the *ELN* cDNA sequence (195 bp product). Standard PCR conditions were used: 95°C for 2 min; 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, with an extension of 5 min at 72°C. The PCR bands were gel purified and sequenced by fluorescent dye terminator cycle sequencing. The integrity of the RNA was assessed by PCR analysis of the human β-actin gene (330 bp product) using the same cycling conditions.

Primer sequences: *ELN* X30F, 5′ GCT GGG CTC GGA GGA CTC GGA GTC; *ELN* X33R, 5′ GGG AAA ATG GGA GAC AAT CCG AAG; β-actin F: 5′ GGC CGT CTT CCC CTC CAT C; β-actin R: 5′ TAG CAA CGT ACA TGG CTG GGG.

Quantitation of mRNA

mRNA was prepared from total RNA (BCL mRNA kit). Slot-blot hybridization filters were prepared using 4 and 0.5 μg of mRNA from AS and two controls, aliquoted in duplicate onto Hybond N+ and hybridized with cDNA probes according to the manufacturer's instructions (Amersham). PCR products from within exons 30–33, exons 1–2 of the *ELN* cDNA and a 330 bp fragment from the human β-actin gene were used as probes. The levels of mRNA were measured densitometrically using instant imager equipment.

Radiolabelling and immunoprecipitation of tropoelastin

Duplicate low passage normal and patient dermal fibroblast cultures were grown to confluency then incubated for 18 h in the presence of 250 mCi of ^{14}C-labelled amino acids per confluent 75 cm^3 flask. Proteinase inhibitors were added and cell layers extracted sequentially in 0.05 M Tris–HCl, pH 7.4 containing 0.2 M NaCl and 1% (v/v) Nonidet P-40 (NET buffer), and 0.05 M Tris–HCl, pH 7.4 containing 4 M guanidine-HCl. Medium and cell layer fractions were pre-cleared with protein A–Sepharose and tropoelastin was immunoprecipitated using a polyclonal tropoelastin antibody (Universal Biologicals). Immunoprecipitates were separated on 8% SDS–PAGE gels.

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