Journal of Cell Science 108, 1317-1323 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

Cysteine-to-arginine point mutation in a 'hybrid' eight-cysteine domain of FBN1: consequences for fibrillin aggregation and microfibril assembly

Cay M. Kielty[1,*], Terhi Rantamäki[2], Anne H. Child[3], C. Adrian Shuttleworth[1] and Leena Peltonen[2]

[1]School of Biological Sciences, Stopford Building 2.205, University of Manchester, Oxford Road, Manchester M13 9PT, UK
[2]Department of Human Molecular Genetics, National Public Health Institute, FIN-00300 Helsinki, Finland
[3]Heart Science Centre and National Heart and Lung Institute, Harefield Hospital, Middlesex UB9 6JH, UK

*Author for correspondence

SUMMARY

Mutations in the FBN1 gene encoding the microfibrillar glycoprotein fibrillin cause Marfan syndrome, a relatively common autosomal dominant connective tissue disease. Causative FBN1 mutations appear to be dispersed throughout the coding frame, and to date no predictable genotype : phenotype correlations have emerged. We have identified a point mutation within an eight-cysteine 'hybrid' motif of the fibrillin polypeptide which results in the substitution of an arginine for a cysteine, in a patient severely affected in the cardiovascular, skeletal and ocular systems.

We have utilised cell cultures from various tissues of this patient to investigate the effects of this mutation on fibrillin expression and deposition, and the consequences in terms of microfibril assembly and organisation. We have established that there is no difference in the expression of normal and mutant alleles, and fibrillin synthesis, secretion and deposition are also normal. However, the rate of fibrillin aggregation is reduced and microfibrillar assemblies are both remarkably scarce and morphologically abnormal.

These data clearly demonstrate that the mutated allele interferes with normal assembly, and strongly implicate this particular region of the fibrillin-1 molecule in stabilising microfibrillar assemblies.

Key words: Marfan syndrome, fibrillin, FBN1 mutation, abnormal microfibril

INTRODUCTION

It is now well documented that mutations in the FBN1 gene that encodes the glycoprotein fibrillin cause Marfan syndrome, a relatively common heritable connective tissue disorder characterised by cardiovascular, skeletal and ocular manifestations (Lee et al., 1991; Kainulainen et al., 1992; Tsipouras et al., 1992; Pyeritz, 1993). Fibrillin is the principal structural element of a class of connective tissue microfibrils that have a widespread distribution (Sakai et al., 1986, 1991; Keene et al., 1991; Kielty et al., 1991, 1993; Wallace et al., 1991). They are particularly abundant in tissues affected in Marfan syndrome, such as aorta, periosteum, ciliary zonules, the alveolar wall and skin. In elastic tissues, fibrillin microfibrils play a key role in elastic fibrillogenesis and are components of elastic fibres which generate elastic recoil (Cleary and Gibson, 1983; Mecham and Heuser, 1991). In non-elastic tissues, they are proposed to play an anchoring role (Ramirez et al., 1993).

Ultrastructural analyses have highlighted the structural complexity of fibrillin microfibrils (Wright and Mayne, 1988; Fleischmajer et al., 1991; Keene et al., 1991; Kielty et al., 1991). By rotary shadowing electron microscopy, they exhibit a 'beads-on-a-string' appearance, with an average beaded periodicity of 55 nm and diameter of 10-12 nm. The mechanism of extracellular assembly and organisation of fibrillin-1 within microfibrils is undoubtedly complex and remains to be defined. The recent cloning and sequencing of a second fibrillin gene, FBN2, and the identification of several microfibril-associated glycoproteins have raised the possibility that microfibrils are multi-component assemblies (Gibson et al., 1991; Horrigan et al., 1992; Zhang et al., 1994).

Marfan syndrome has a high penetrance, but is characterised by strikingly variable inter- and intra-familial phenotypes (Pyeritz, 1993). Recently, attempts have been made to derive predictable genotype : phenotype correlations as a means of understanding the pathogenesis of Marfan syndrome. Mutation analyses have successfully identified more than thirty distinct mutations dispersed throughout the coding sequence of FBN1 to date (Dietz et al., 1991, 1992a,b, 1993a,b; Kainulainen et al., 1992, 1994; Godfrey et al., 1993; Hewitt et al., 1993; Tynan et al., 1993; Hayward et al., 1994; Milewicz and Duvic, 1994). Concurrently, biochemical analyses have highlighted variations between patient cells with respect to synthesis, secretion and deposition of fibrillin (Milewicz et al., 1992; Aoyoma et al., 1994; Kielty and Shuttleworth, 1994). However, no clearcut connection between mutation and disease phenotype has emerged, which highlights the complexity of fibrillin microfibrils in terms of their composition, organisation and biological interactions.

We have recently applied a combination of biochemical and ultrastructural approaches to investigate how defined fibrillin mutations influence microfibril formation and organisation

(Kielty and Shuttleworth, 1994; Kielty et al., 1994). These studies have highlighted a range of microfibrillar abnormalities and differences in fibrillin expression and aggregation between Marfan patient cell lines. To gain insights into Marfan genotype : phenotype relationships, we have extended this strategy to investigate fibrillin expression and assembly using cell cultures derived from skin and cardiovascular tissues of a severely affected Marfan patient whose causative mutation is reported here.

MATERIALS AND METHODS

Clinical history

The patient DG was affected in all three systems. His height was 193 cm, with an arm span of 210 cm and lower segment of 106 cm. He had arachnodactyly with small joint hypermobility. He had a large forehead with no dolichocephaly, a broad palate, but no scoliosis or pectus deformity. He had bilateral dislocated lenses, with no antimongoloid slant. Prior to aortic root replacement, his left ventricle was dilated with moderate left ventricular function. He had posterior mitral valve prolapse with mild to moderate mitral regurgitation. His left atrium was 4.1 cm. The ascending aorta was dilated with aortic annulus 2.9 cm, aortic cusp tip diameter 5.2 cm, aortic maximum diameter 6.2 cm and mild to moderate aortic regurgitation. Tricuspid regurgitation was present. There was no evidence of dissection. His aortic root and mitral valve were replaced at operation, he developed infection and congestive heart failure following the operation, and died approximately two weeks post-operation at the age of 45.

Patient material

Cell lines from three different tissue sites were established by explant. Dermal fibroblasts were established from skin biopsies, aortic cusp cells and aortic wall cells and mitral valve cells from tissue were removed at operation. Blood samples were taken from the proband and relatives in accordance with the Helsinki Declaration. Diagnosis was made using published criteria.

Mutation analysis

Fibrillin specific primers that were used in single-stranded conformation analysis (SSCA) have previously been published (Kainulainen et al., 1994). In addition, the following primers were used in SSCA, solid-phase sequencing and solid-phase minisequencing: (a) nt 2462-2483; (b) nt 2540-2561; (c) nt 2650-2667; (d) nt 2736-2713 (5′-biotinylated); (e) 5′-CAAAGACCATTGGAGTGGTATAG-3′ (intronic sequence, 5′-biotinylated); nt numbering in accordance with Pereira et al. (1993).

Genomic DNA was extracted from peripheral leucocytes of the family members of patient DG as well as control individuals as previously described (Vandenplas et al., 1984). Total RNA and genomic DNA of 60 Marfan patients were extracted from cultured fibroblasts using the GIT-CsCl method with modifications (Chirgwin et al., 1979). Reverse transcription reactions and amplification of cDNA in overlapping fragments covering the FBN1 cDNA sequence, and PCR conditions for SSCA and sequencing, were carried out as previously described (Kainulainen et al., 1992).

SSCA of amplified cDNAs was carried out at room temperature using 5% acrylamide (acrylamide/bisacrylamide, 60/1, w/w) gels containing 10% glycerol.

Both genomic and cDNA of patient DG were sequenced with primer b using solid-phase sequencing with slight modifications (Syvänen et al., 1989).

Determination of steady-state transcript levels

The steady-state levels of transcribed FBN1 alleles relative to the amount of genomic DNA were determined using a solid-phase minisequencing method (Ikonen et al., 1992). PCR of genomic DNA and cDNA was carried out with primers b-e and b-d, respectively, with an annealing temperature of 56°C. PCR products were attached to a streptavidin-coated microtitre well by passive absorbtion and denatured with 50 mM NaOH. The primer annealing and extension reactions were carried out simultaneously by incubating the wells at 50°C for 10 minutes in the reaction mixture containing 10 pmol primer c, 0.5 μCi [^{3}H]dNTP (either CTP or TTP), 0.25 U *Taq* DNA polymerase in a buffer (20 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 15 mM $(NH)_4SO_4$, 0.01% gelatin, 0.1% Tween-20). The extended primer was released with 50 mM NaOH and the eluted radioactivity was measured in a liquid scintillation counter. The ratio (*R*) between the incorporated nucleotide [^{3}H]CTP corresponding to the mutated allele and [^{3}H]TTP corresponding to the normal allele was calculated (Syvänen et al., 1990).

Cells and cell culture

Cells were routinely maintained in Dulbecco's minimum essential medium supplemented with 10% foetal calf serum, penicillin (400 i.u./ml), streptomycin (50 mg/ml), and glutamine (200 mg/ml). Confluent cells were pulse-labelled for 30 minutes or continuously labelled for 16 hours or 72 hours with [^{35}S]tranS label in medium containing 0.5% foetal calf serum. Proteinase inhibitors (2 mM phenylmethanesulphonyl fluoride (PMSF) and 5 mM *N*-ethylmaleimide (NEM)) were then added. Cell layers were sequentially extracted in 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl and 1% (v/v) Nonidet P40 (NNT buffer), and 0.05 M Tris-HCl, pH 7.4, containing 0.4 M guanidine-HCl. Fibrillin was immunoprecipitated from medium and cell layer compartments as previously described (Kielty et al., 1993, 1994).

For microfibril extractions, cells were maintained at post-confluence for up to two weeks. Cell layers were washed in 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl and 0.005 M $CaCl_2$, then incubated for 2 hours at 20°C with 0.1 mg/ml bacterial collagenase (type 1A), 2 mM PMSF and 5 mM NEM. Soluble extracts were clarified by centrifugation for 15 minutes at 7,500 ***g*** on a bench microfuge before size fractionation by gel filtration chromatography on Sepharose CL-2B (Kielty et al., 1991).

Immunoprecipitation

Immunoprecipitation was carried out on medium in 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl, 0.005 M EDTA and 1% (v/v) Nonidet P40 (NET buffer) essentially as described by Kielty et al. (1990). In view of the similar electrophoretic mobilities on SDS-PAGE of fibronectin and fibrillin, fibronectin was removed before immunoprecipitation of fibrillin by two sequential incubations with 100 μl of a 1:1 (v/v) solution of gelatine-Sepharose. Samples were then incubated for 1 hour at 20°C with a 1:100 dilution of a polyclonal fibrillin antiserum (Kielty et al., 1993, 1994; Raghunath et al., 1994), before the addition of 60 μl of a 1:1 (v/v) solution of Protein A-Sepharose in NET buffer.

Size fractionation

Metabolically labelled fibrillin immunoprecipitated from normal and DG dermal fibroblast culture medium was chromatographed on an analytical gel filtration column (1 cm × 17 cm) of Sepharose CL-2B. The column was equilibrated and eluted at room temperature with 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl. In each run, 60 × 0.2 ml fractions were collected. The elution positions of newly synthesised, metabolically labelled fibrillin were determined by counting each fraction.

Electrophoresis

Immunoprecipitates were separated by discontinuous SDS-PAGE on 8% gels (Laemmli, 1970) under non-reducing conditions, then analysed by fluorography. Molecular masses were determined by

reference to prestained standards (fumarase (M_r 56,000) pyruvate kinase (M_r 65,000) fructose-6-phosphate kinase (M_r 88,000), β-galactosidase (M_r 125,000), α_2-macroglobulin (M_r 190,000)).

Ultrastructural analysis

Void volume fractions of cell layer extracts were visualised for their microfibril content by rotary shadowing using a modification of the mica sandwich technique (Kielty et al., 1994). Rotary shadowing immunoelectron microscopy was carried out as previously described (Kielty et al., 1994).

RESULTS

Low passage cultured cells derived from skin, aortic cusp and aortic wall of the patient DG were used in these investigations.

Mutation analysis

The complete coding region of FBN1 cDNA was screened for nucleotide changes in amplified overlapping fragments using the SSCA method as previously described in detail (Kainulainen et al., 1994). A mobility shift in a sample of patient DG was identified in SSCA when cDNA was amplified with primers a-d (Fig. 1A). Sequencing of the corresponding cDNA and genomic DNA revealed a point mutation of T to C at nucleotide 2668 resulting in a substitution of arginine for cysteine (C890R) in a 'hybrid' 8-cysteine domain of the fibrillin polypeptide (Fig. 1B). The mutation was also identified in the samples of living affected family members of patient DG but not in any samples of unaffected family members. A panel of DNAs from 60 unrelated Marfan patients and a panel of 200 unrelated control individuals were screened for the heterozygous C890R mutation using the solid-phase minisequencing method, and all the patients in the panels were found to carry the normal nucleotide (T) in both alleles.

Normal and mutant transcript levels

To determine the level of transcription of the mutated allele the *R*-value obtained from solid-phase minisequencing test of cDNA representing the ratio between mutant and normal mRNA was compared to the *R*-value of the genomic DNA in which the alleles are in a 1:1 ratio. The minisequencing analysis was carried out three times from independent reverse transcription reactions and samples of genomic DNA of patient DG following independent PCR reactions of cDNA and genomic DNA. The *R*-value of cDNA was observed to range from 1.06 to 1.27 and the corresponding *R*-value for the genomic DNA varied from 1.00 to 1.38. This indicates that there is no difference in the steady-state levels of the mutated and normal transcripts.

Table 1. Distribution of counts incorporated into newly synthesised fibrillin by DG cell lines

Cell line	%Total incorporated counts in fibrillin	Distribution of fibrillin, % in:	
		Medium	Cell layer
DG dermal fibroblasts	0.128	29	71
DG aortic cusp cells	0.115	44	56
DG aortic valve cells	0.102	41	59
Normal dermal fibroblasts	0.142	36	64

Cells were labelled for 16 hours with [^{35}S]tranS label. Fibrillin was immunoprecipitated from medium and cell layer extracts of normal fibroblast culture, DG skin fibroblasts, aortic cusp cells, and aortic valve cell lines. The amount and distribution of newly synthesised fibrillin was compared by determining the percentage of total immunoprecipitated counts in medium and cell layer extracts. The values are averages from two duplicate experiments.

Fibrillin expression and deposition

The expression and deposition of fibrillin was investigated after labelling and immunoprecipitation of fibrillin from medium, cell lysate and cell layer fractions of dermal fibroblasts from a normal control, and dermal fibroblasts, aortic cusp cells and aortic wall cells from patient DG. De novo fibrillin synthesis was expressed as total counts incorporated into fibrillin (Table 1). Of the DG cell lines, the dermal fibroblasts synthesised highest levels of fibrillin. In all cases, the majority of incorporated counts could be found in cell layers.

The electrophoretic mobility and time-course of secretion and deposition of fibrillin were determined in pulse-chase labelling of dermal fibroblasts, and 16 hours and 72 hours continuous labelling experiments using normal dermal fibroblasts and the three DG cell lines (Fig. 2). Metabolically labelled fibrillin was immunoprecipitated from medium after 2 hours, 6 hours and 16 hours chases, and analysed on SDS-PAGE gels under non-reducing conditions (Fig. 2A). Two fibrillin-immunoreactive components (M_r 300,000 and 330,000) were present in the medium at 2 hours and in abundance by 6 hours,

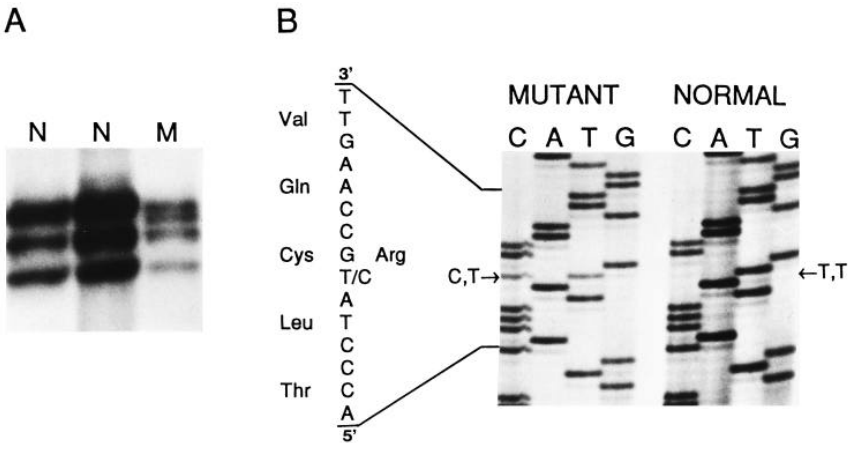

Fig. 1. (A) SSCA of amplified fibrillin cDNA from patient DG (third lane) and two other Marfan patients. A differently migrating band is seen in patient DG's lane (N, an unrelated Marfan patient; M, patient DG). (B) Sequence analysis of genomic DNA showing the heterozygous T to C mutation of the patient DG at nt 2668 resulting in the substitution of arginine for cysteine (C890R).

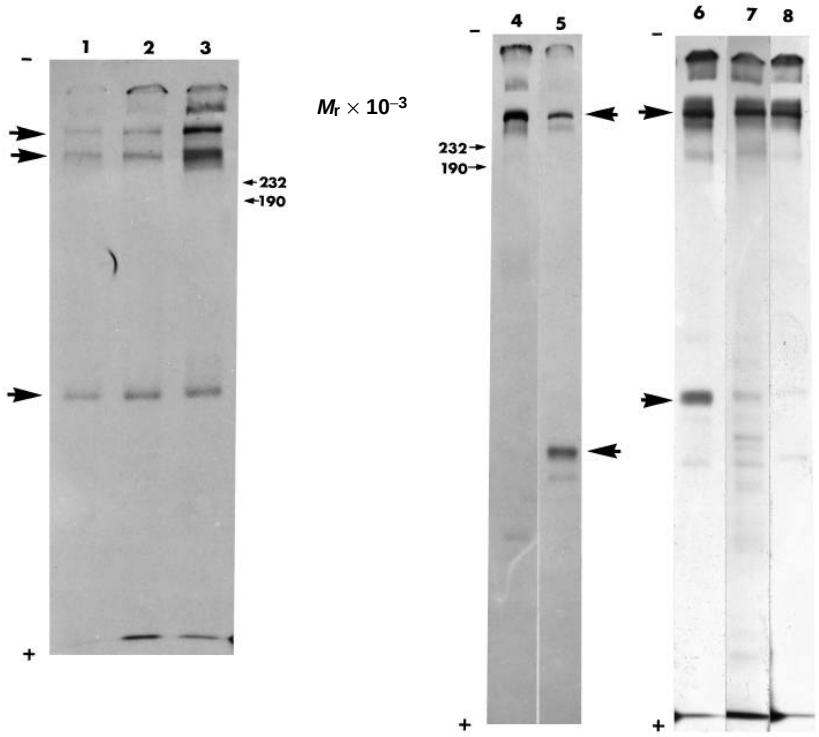

Fig. 2. Electrophoretic analysis of fibrillin immunoprecipitated from medium of DG dermal fibroblasts, aortic cusp cells, and aortic valve cells. Cells were either pulse-labelled with [^{35}S]tranS label for 30 minutes and chased for 2 hours, 6 hours and 16 hours, or continuously labelled for 16 hours or 72 hours. Samples were analysed by SDS-PAGE on 8% gels under non-reducing conditions and by fluorography. The electrophoretic mobilities of relative molecular mass markers catalase (M_r 232,000) and α_2-macroglobulin (M_r 190,000) are indicated. In the pulse-chase, fibrillin-immunoreactive bands of M_r 300,000 and 330,000 were present in the medium after 2 hours chase and in abundance by 16 hours chase. At 16 hours chase, the M_r 300,000 component resolved as a doublet. By 6 hours, there was some evidence for formation of higher-M_r aggregates that failed to enter the gel, but little further aggregation was apparent. A fibrillin-immunoreactive component (M_r 55,000) was also detected at all time points. When DG dermal fibroblasts, aortic cusp cells and aortic valve cells were continuously labelled for 16 hours or 72 hours, a major fibrillin-immunoreactive band corresponding to M_r 300,000 was always detected, together with large aggregates and a smaller component. Tracks 1-3, pulse-chase immunoprecipitates; tracks 4-8, continuous labelling immunoprecipitates. Track 1, 2 hours chase; track 2, 6 hours chase; track 3, 16 hours chase. Track 4, 16 hours label, normal dermal fibroblasts; track 5, 16 hours label, DG dermal fibroblasts; track 6, 72 hours label, DG dermal fibroblasts; track 7, 72 hours label, DG aortic cusp cells; track 8, 72 hours label, DG aortic wall cells. Fibrillin-immunoreactive components are indicated by large arrowheads.

and there was some evidence by 6 hours for the formation of higher-M_r intermediate assemblies and larger aggregates which did not enter the gel. However, not all of the newly synthesised fibrillin had been incorporated into aggregates by 16 hours, in marked contrast to normal cultures (Kielty et al., 1993, 1994). After 16 hours or 72 hours continuous labelling, a major component (M_r 300,000) and higher-M_r aggregates were immunoprecipitated from control and DG culture medium (Fig. 2B). There was also evidence in DG medium for a smaller-M_r 55,000 fibrillin-immunoreactive component which may be a fibrillin degradation product.

Assembly

Formation of high-M_r aggregates containing newly synthesised

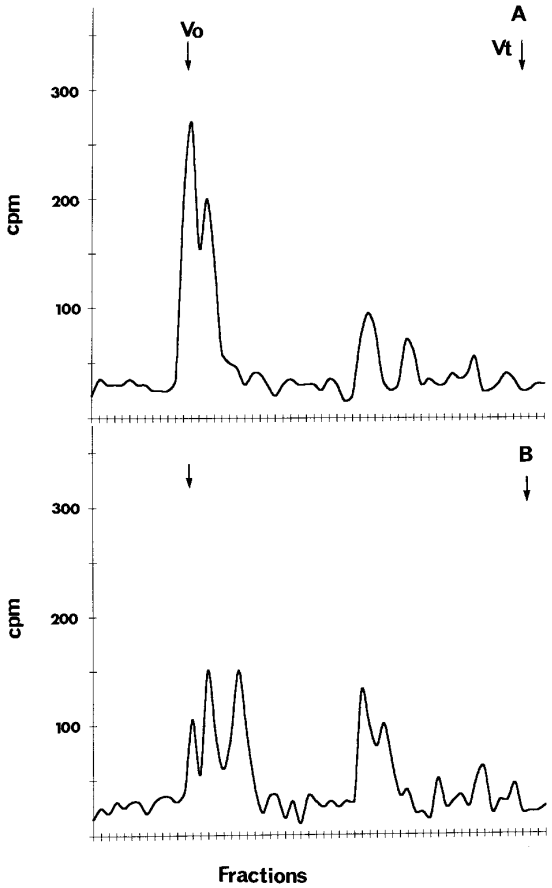

Fig. 3. Size fractionation of newly synthesised fibrillin immunoprecipitated from the medium of DG and normal dermal fibroblasts. Cells were continuously labelled with [^{35}S]tranS label for 16 hours. Immunoprecipitated newly synthesised fibrillin was size fractionated on an analytical Sepharose CL-2B column, and elution profiles were determined by analysing the distribution of fibrillin counts. The elution positions of V_o and V_t are indicated. In control culture medium, the majority of newly synthesised fibrillin was present as high-M_r aggregates. In the patient culture medium, in contrast, whilst some newly synthesised fibrillin was present as high-M_r aggregates, there were also prominent peaks of smaller fibrillin-immunoreactive material which could correspond to unpolymerised monomers and intermediate assemblies. (A) Fibrillin immunoprecipitated from control dermal fibroblast culture medium; (B) fibrillin immunoprecipitated from patient dermal fibroblast culture medium.

fibrillin within medium and cell layers of normal and DG skin fibroblasts was demonstrated by size fractionation of labelled fibrillin immunoprecipitated from medium on Sepharose CL-2B (Fig.3). Fibrillin immunoprecipitated from normal and patient cell medium after 16 hours chases showed aggregation within the M_r range 3×10^5 to 5×10^6. The rate of aggregation of newly synthesised fibrillin in the patient cultures was markedly slower than in the control culture.

Ultrastructural analysis

Examination by rotary shadowing electron microscopy of high relative molecular mass material solubilised from post-confluent cell layers of DG dermal fibroblasts, aortic cusp cells, and aortic wall cells revealed that morphologically identifiable microfibrillar assemblies were remarkably sparse. In contrast, normal fibroblasts elaborated extensive microfibrils similar in morphology and periodicity to those previously isolated from

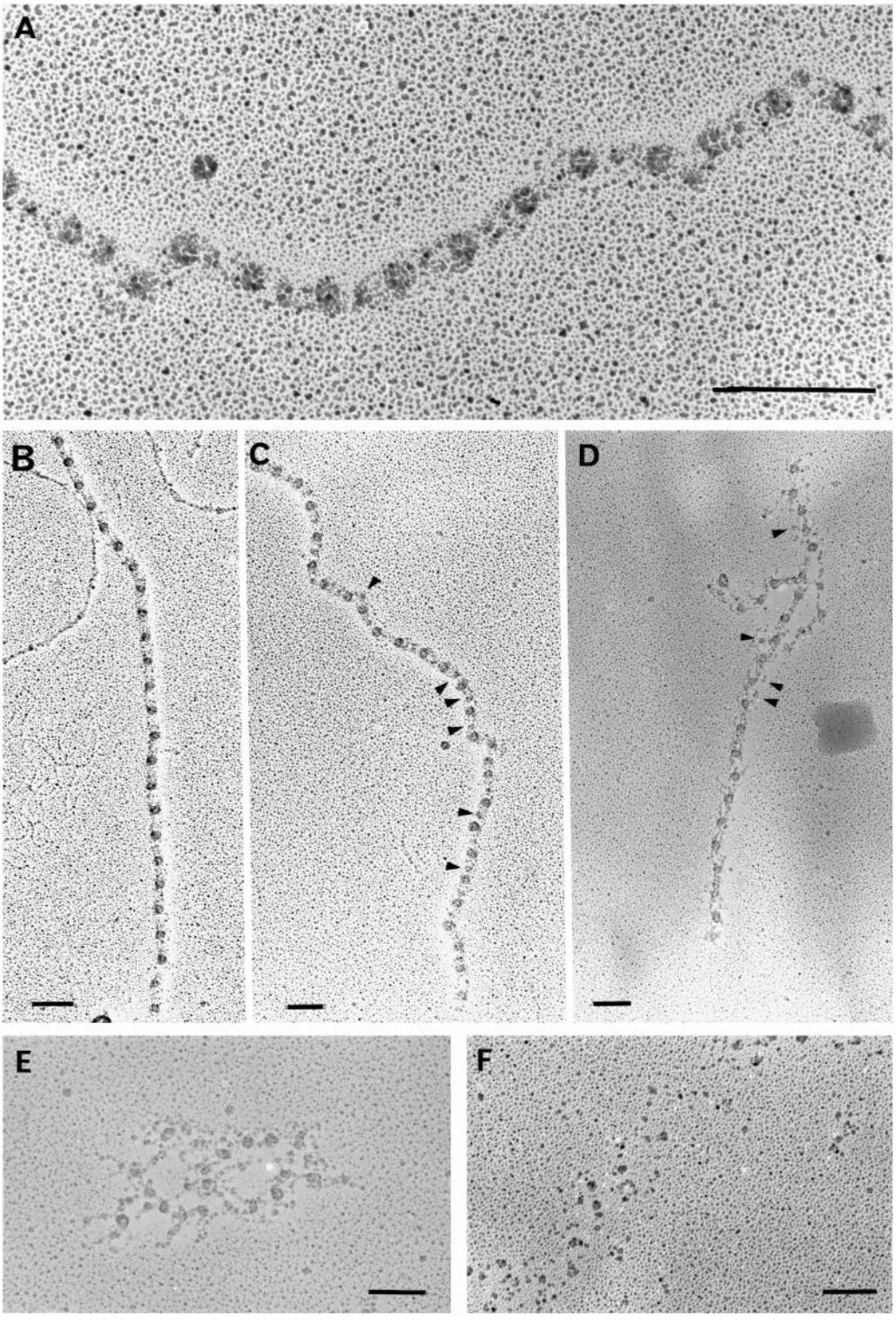

Fig. 4. Rotary shadowing electron micrographs of microfibrils isolated from normal and patient dermal fibroblast cell layers. DG dermal fibroblasts and aortic wall cells elaborated very few morphologically identifiable microfibrillar assemblies. Those observed had an average periodicity of 48 nm and exhibited marked abnormalities in the form of abnormal electron-dense regions within interbead domains (see arrowheads). Treatment with EDTA or DTT grossly disrupted microfibril organisation.
(A and C) Microfibrils elaborated by DG skin fibroblasts. (B) Microfibrils elaborated by normal dermal fibroblasts. (D) Microfibrils elaborated by DG aortic wall cells. (E and F) DG skin fibroblast microfibrils treated with 5 mM EDTA and 10 μM DTT, respectively, for 1 minute prior to freeze-drying on mica. Bars, 100 nm.

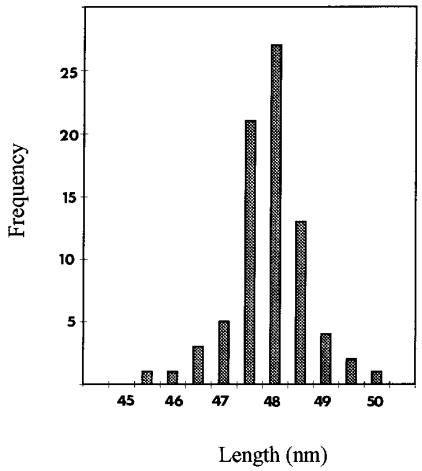

Fig. 5. Histogram demonstrating the periodicity of fibrillin microfibrils elaborated by cultured dermal fibroblasts from patient DG. Microfibrils were isolated from post-confluent DG fibroblasts cell layers and visualised by rotary shadowing electron microscopy as previously described (Kielty et al., 1994). Microfibrillar periodicity was 48 nm (range 45-50 nm).

tissues (Kielty et al., 1991, 1993) (Fig. 4B). Only a few microfibrils were detected in the DG dermal fibroblast and aortic wall cell cultures, and these had a reduced average periodicity (Fig. 4A, C, D; Fig. 5). Their interbeaded domains were poorly packed and there also appeared to be an electron-dense bead-like organisation associated with these domains. Treatment with 5 mM EDTA and 10 μM DTT rapidly disrupted these microfibrillar assemblies (Fig. 4E,F). In addition, loose fibrillin aggregates that lacked normal microfibrillar morphology were present and identified immunologically (not shown).

DISCUSSION

The relationship between defined mutations in FBN1, fibrillin defects, microfibrillar abnormalities and clinical phenotype is still not understood, despite the recent accumulation of mutation and biochemical data. To date, the only demonstrable correlation between genotype and phenotype occurs at the beginning of the longest stretch of EGF repeats in the middle of the fibrillin molecule, where mutations resulting in a neonatal, severe form of Marfan syndrome seem to cluster (Kainulainen et al., 1994; Milewicz and Duvic, 1994). However, so far the presence of mutated fibrillin polypeptides has been demonstrated directly in only a few cases (Kainulainen et al., 1992; Raghunath et al., 1994).

The substitution of arginine for cysteine within a hybrid 8-cysteine domain reported here is predicted to knock out a disulphide bond (intra- or inter-molecular), which will inevitably alter domain conformation, whilst the introduction of a positive charge may cause repulsion between adjacent monomers. We have used a cell culture system to investigate the effects of this novel FBN1 mutation at the levels of synthesis, secretion and deposition, and the consequences for microfibril organisation. This is the first report where data have been obtained using three cell lines from distinct affected tissues of an individual patient. These lines differed only in the amount of fibrillin they express.

Although the expression and secretion of mutated fibrillin in the patient dermal fibroblasts was comparable with that in normal cells, a reduced rate of fibrillin aggregation was apparent in the patient cultures and the presence of a lower-M_r fibrillin-immunoreactive component suggested some fibrillin degradation. When fibrillin assembly was investigated at the ultrastructural level, it became clear that few morphologically identifiable microfibrils had been elaborated when compared with other normal and Marfan cell lines previously examined (Kielty and Shuttleworth, 1994; Kielty et al., 1993, 1994), and where detected these microfibrils were manifestly abnormal. There were also abundant loose fibrillin aggregates that were identified immunologically but not morphologically. These results clearly demonstrate that secreted mutant allele products disrupt normal assembly. This is the first direct evidence to implicate this hybrid motif in the formation and stabilisation of microfibrillar assemblies.

Our results demonstrate conclusively that fibrillin assemblies in these cell cultures are structurally and functionally abnormal. The spectrum of severe clinical manifestations of this patient indicate that his fibrillin microfibrils failed to function normally in their elastic fibre context, and also where they occur in skeletal and ocular tissues as loose microfibril bundles devoid of elastin. The likelihood is that an intrinsic structural and/or mechanical microfibrillar property has been compromised, although interaction with tropoelastin appears not to be directly affected. In view of the exclusively microfibrillar composition of the ciliary zonules, the bilateral lens dislocation suffered by this patient provides supportive evidence that this is, indeed, the case. It has always been considered that ectopia lentis is due to a loss of elasticity of the zonular fibres (Nelson and Maumanee, 1982) and, interestingly, ultrastructural analyses have suggested that normal fibrillin microfibrils possess the capacity to extend and retract (Keene et al., 1991). In the case of our patient, however, the microfibrils were clearly unable to sustain their role in the dynamic suspension of the lens. One can speculate that a template of disrupted, fragmented microfibrils may have generated elastic fibres that, in maturity, exhibited progressive inability to sustain their elastic function, resulting in catastrophic failure within the cardiovascular system. It is of interest that the only other Marfan syndrome mutation reported in this region (C862R) also results in a relatively severe phenotype with bilateral lens luxation and cardiovascular symptoms requiring aortic surgery (Tynan et al., 1994).

This is the first such comprehensive assessment of the relationship between genotype and phenotype for an individual patient. It has highlighted the crucial contribution of the second hybrid 8-cysteine motif of fibrillin-1 to normal structure and function.

This work was supported by the Medical Research Council and The Academy of Finland. A.H.C. thanks the British Heart Foundation and the Heart Science Centre (National Heart and Lung Institute, London) for their support.

REFERENCES

Aoyama, T., Tynan, K., Dietz, H. C., Francke, U. and Furthmayr, H.

(1994). Missense mutations impair intracellular processing of fibrillin and microfibril assembly in Marfan syndrome. *Hum. Mol. Genet*. **2**, 2135-2140.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294-5299.

Cleary, E. G. and Gibson, M. A. (1983). Elastin-associated microfibrils and microfibrillar proteins. *Int. Rev. Connect. Tiss. Res*. **10**, 97-209.

Dietz, H. C., Cutting, G. R., Pyeritz, R. E., Maslen, C. L., Sakai, L. Y., Corson, G. M., Puffenberger, E. G., Hamosh, A., Nanthakumar, E. J., Curristan, S. M., Stetten, G., Meyers, D. A. and Francomano, C. A. (1991). Marfan syndrome caused by a recurrent de novo missense mutation in the fibrillin gene. *Nature* **352**, 337-339.

Dietz, H. C., Saraiva, J. M., Pyeritz, R. E., Cutting, G. R. and Francomano, C. A. (1992a). Clustering of fibrillin (FBN1) missense mutations in Marfan syndrome patients at cysteine residues in EGF-like domains. *Hum. Mutat*. **1**, 366-374.

Dietz, H. C., Pyeritz, R. E., Puffenberger, E. G., Kendzior, R. J., Corson, G. M., Maslen, C. L., Sakai, L. Y., Francomano, C. A. and Cutting, G. R. (1992b). Marfan phenotype variability in a family segregating a missense mutation in the epidermal growth factor-like motif of the fibrillin gene. *J. Clin. Invest.* **89**, 1674-1680.

Dietz, H. C., Valle, D., Francomano, C. A., Kendzior, R. J., Pyeritz, R. E. and Cutting, G. R. (1993a). The skipping of constitutive exons in vivo induced by nonsense mutations. *Science* **259**, 680-683.

Dietz, H. C., McIntosh, I., Sakai, L. Y., Corson, G. M., Chalberg, S. C., Pyeritz, R. E. and Francomano, C. A. (1993b). Four novel FBN1 mutations: significance for mutant transcript level and EGF-like domain calcium binding in the pathogenesis of Marfan syndrome. *Genomics* **17**, 468-475.

Fleischmajer, R., Contard, P., Schwartz, E., MacDonald, E. D., Jacobs, L. and Sakai, L. Y. (1991). Elastin-associated microfibrils (10 nm) in a three-dimensional fibroblast culture. *J. Invest. Dermatol*. **97**, 639-643.

Gibson, M. A., Sandberg, L. B., Grosso, L. E. and Cleary, E. G. (1991). Complementary DNA cloning establishes microfibril-associated glycoprotein (MAGP) to be a discrete component of elastin-associated microfibrils. *J. Biol. Chem*. **266**, 7596-7601.

Godfrey, M., Vandemark, N., Wang, M., Vellnov, M., Wargowski, D., Droste, S. and Rao, V. H. (1993). Prenatal diagnosis and a donor splice site mutation in fibrillin in a family with Marfan syndrome. *Am. J. Hum. Genet*. **49**, 662-667.

Hayward, C., Porteous, M. E. M. and Brock, D. J. H. (1994). Identification of a novel nonsense mutation in the fibrillin gene (FBN1) using nonisotopic techniques. *Hum. Mutat.* **3**, 159-162.

Hewitt, D. R., Lynch, J. R., Smith, R. and Sykes, B. (1993). A novel fibrillin mutation in the Marfan syndrome which could disrupt calcium binding of the epidermal growth factor-like module. *Hum. Mol. Genet.* **2**, 475-477.

Horrigan, S. K., Rich, C. B., Streeten, B. W., Li, Z. Y. and Foster, J. A. (1992). Characterisation of an associated microfibril protein through recombinant DNA techniques. *J. Biol. Chem.* **267**, 10087-10095.

Ikonen, E., Manninen, T., Peltonen, L. and Syvänen, A-C. (1992). Quantitative determination of rare mRNA species by PCR and solid-phase minisequencing. *PCR Meth. Applicat.* **1**, 234-240.

Kainulainen, K., Sakai, L. Y., Child, A. H., Pope, F. M., Puhakka, L., Ryhanen, L., Palotie, A., Kaitila, I. and Peltonen, L. (1992). Two mutations in Marfan syndrome resulting in truncated fibrillin polypeptides. *Proc. Nat. Acad. Sci. USA* **89**, 5917-5921.

Kainulainen, K., Karttunen, K., Puhakka, L., Sakai, L. and Peltonen, L. (1994). Mutations in the fibrillin gene responsible for dominant ectopia lentis and neonatal Marfan syndrome. *Nature Genet.* **6**, 64-69.

Keene, D. R., Maddox, B. K., Kuo, H.-J., Sakai, L. Y. and Glanville, R. W. (1991). Extraction of extendable beaded structures and their identification as fibrillin-containing extracellular microfibrils. *J. Histochem. Cytochem*. **39**, 441-449.

Kielty, C. M., Boot-Handford, R. P., Ayad, S., Shuttleworth, C. A. and Grant, M. E. (1990). Molecular composition of type VI collagen. *Biochem J*. **272**, 787-795.

Kielty, C. M., Cummings, C., Whittaker, S. P., Shuttleworth, C. A. and Grant, M. E. (1991). Isolation and ultrastructural analysis of microfibrillar structures from foetal bovine elastic tissues. *J. Cell Sci*. **99**, 797-807.

Kielty, C. M., Berry, L., Whittaker, S. P., Grant, M. E. and Shuttleworth, C. A. (1993). Microfibrillar assemblies of foetal bovine skin. *Matrix* **13**, 103-112.

Kielty, C. M. and Shuttleworth, C. A. (1994). Abnormal fibrillin assembly by dermal fibroblasts from two patients with Marfan syndrome. *J. Cell Biol.* **124**, 997-1004.

Kielty, C. M., Phillips, J. E., Child, A. H., Pope, F. M. and Shuttleworth, C. A. (1994). Fibrillin secretion and microfibril assembly by Marfan dermal fibroblasts. *Matrix Biol.* **14**, 191-199.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lee, B., Godfrey, M., Vitale, E., Hori, H., Mattei, M.-G., Sarfarazi, M., Tsipouras, P., Ramirez F. and Hollister, D. W. (1991). Linkage of Marfan syndrome and a phenotypically related disorder to two different fibrillin genes. *Nature* **352**, 330-334.

Mecham, R. P. and Heusar, J. E. (1991). The elastic fiber. In *Cell Biology of the Extracellular Matrix*, 2nd edn (ed. E. D. Hay), pp. 79-109. Plenum Publishing Co., New York.

Milewicz, D. M., Pyeritz, R. E., Crawford. E. S. and Byers, P. H. (1992). Marfan syndrome: defective synthesis, secretion and extracellular matrix formation of fibrillin by cultured dermal fibroblasts. *J. Clin. Invest*. **89**, 79-86.

Milewicz, D. M. and Duvic, M. (1994). Severe neonatal Marfan syndrome resulting from a *de novo* 3-bp insertion into the fibrillin gene on chromosome 15. *Am. J. Hum. Genet*. **54**, 447-453.

Nelson, L. B. and Maumanee, I. H. (1982). Ectopia lentis. *Surv. Ophthalmol.* **27**, 143-160.

Pereira, L., D'Alessio, M., Ramirez, F., Lynch, J. R., Sykes, B., Pangilinan, T. and Bonadio, J. (1993). Genomic organisation of the sequence coding for fibrillin, the defective gene product in Marfan syndrome. *Hum. Mol. Genet.* **2**, 961-968.

Pyeritz, R. E. (1993). The Marfan Syndrome. In *Connective Tissue and its Heritable Disorders* (ed. P. M. Royce and B. Steinmann), pp. 437-468. Wiley-Liss, New York.

Raghunath, M., Kielty, C. M., Kainulainen, K., Child, A., Peltonen, L. and Steinmann, B. (1994). Analyses of truncated fibrillin caused by a 366bp deletion in the FBN1 gene resulting in Marfan syndrome. *Biochem. J*. **302**, 889-896.

Ramirez, F., Pereira, L., Zhang, H. and Lee, B. (1993). The fibrillin-Marfan syndrome connection. *BioEssays* **15**, 589-594.

Sakai, L. Y., Keene, D. R. and Engvall, E. (1986). Fibrillin, a new 350-kD glycoprotein, is a component of extracellular microfibrils. *J. Cell Biol.* **103**, 2499-2509.

Sakai, L. Y., Keene, D. R., Glanville, R. W. and Bachinger, H. P. (1991). Purification and partial characterisation of fibrillin, a cysteine-rich structural component of connective tissue microfibrils. *J. Biol. Chem.* **266**, 14763-14770.

Syvänen, A-C., Aalto-Setala, K., Kontula, K. and Soderlund, H. (1989). Direct sequencing of affinity-captured amplified human DNA: application to the detection of apolipoprotein E polymorphism. *FEBS Lett.* **258,** 71-74.

Syvänen, A-C., Aalto-Setala, K., Harju, L., Kontula, K. and Soderlund, H. (1990). A primer-guided nucleotide incorporation assay in the genotyping of apolipoprotein E. *Genomics* **8**, 684-692.

Tsipouras P.,Del Mastro, R., Sarfarazi, M., Lee, B., Vitale, E., Child, A. H., Godfrey, M., Devereux, R. B., Hewitt, D., Steinmann, B., Viljoen, D., Sykes, B. C., Kilpatrick, M. and Ramirez, F. (1992). Linkage analysis demonstrates that Marfan syndrome, dominant ectopia lentis and congenital contractural arachnodactyly are linked to the fibrillin genes on chromosomes 15 and 5. *New Eng. J. Med*. **326**, 905-909.

Tynan, K.,Comeau, K., Pearson, M., Wilgenbus, P., Levitt, D., Gasner, C., Berg, M. A., Miller, D. C. and Francke, U. (1993). Mutation screening of complete fibrillin-1 coding sequence: report of five new mutations, including two in 8-cysteine domains. *Hum. Mol. Genet*. **2**, 1813-1821.

Vandenplas, S., Wild, I., Grobler-Rabie, A., Brebner, K., Ricketts, M., Wallis, G., Bester, A., Boyd, C. and Mathew, C. (1984). Blot hybridization analysis of genomic DNA. *J. Med. Genet*. **21**, 164-172.

Wallace, R. N., Streeten, B. W. and Hanna, R. B. (1991). Rotary shadowing of elastic system microfibrils in the ocular zonule, vitreous and ligamentum nuchae. *Curr. Eye Res.* **10**, 99-109.

Wright, D. W. and Mayne, R. (1988). Vitreous humor of chicken contains two fibrillar systems: An analysis of their structure. *J. Ultrastruct. Mol. Struct. Res.* **100**, 224-234.

Zhang, H., Apfelroth, S. D., Hu, W., Davis, E. C., Sanguineti, C., Bonadio, J., Mecham, R. P. and Ramirez, F. (1994). Structure and expression of fibrillin-2, a novel microfibrillar component preferentially located in elastic matrices. *J. Cell Biol*. **124**, 855-863.

(Received 20 July 1994 - Accepted 14 November 1994)