Original articles

Marfan syndrome: fibrillin expression and microfibrillar abnormalities in a family with predominant ocular defects

Cay M Kielty, Sally J Davies, Janet E Phillips, Carolyn J P Jones, C Adrian Shuttleworth, Stephen J Charles

Abstract

We have found abnormal fibrillin microfibrils isolated from tissues and cell cultures from two cousins with Marfan syndrome whose major clinical abnormality is bilateral ectopia lentis, but who also have skeletal involvement but no cardiovascular defects. Ultrastructural analysis of ciliary zonules showed the presence of abundant loose microfibril bundles which in many places appeared disorganised. Microfibrils isolated from ciliary zonules and vitreous were highly fragmented when examined by rotary shadowing electron microscopy. Investigation of microfibrils elaborated by patient dermal fibroblasts showed remarkable variations in periodicity and packing. The synthesis and secretion of fibrillin by these cells was confirmed electrophoretically with the identification of metabolically labelled immunoprecipitated fibrillin (M_r 300 000) in medium and cell layer compartments. These data show that fibrillin expression is normal but that assembled microfibrils are manifestly abnormal both morphologically and functionally. The occurrence of microfibrils with variable periodicities and susceptibility to fragmentation suggests that structural weakness is probably the primary cause of lens dislocation in these patients.

(J Med Genet 1995;32:1-6)

Fibrillin is the principle structural element of a class of connective tissue microfibrils that have a widespread distribution.⁴⁻⁹ They are particularly abundant in tissues affected in Marfan syndrome, such as aorta, periosteum, ciliary zonules, the alveolar walls, and skin. Ultrastructural analyses have highlighted the complex architecture of fibrillin microfibrils.^{6,8,10-12} They exhibit a pronounced beaded morphology with a diameter of 10 to 12 nm and an average, but variable, periodicity of 55 nm. The mechanism of fibrillin assembly and microfibril organisation remains to be defined. The molecular complexity of fibrillin microfibrils was recently highlighted with the identification of a second fibrillin (FBN2) locus.¹³

Attempts have been made to relate genotype and phenotype as a means of understanding the pathogenesis of Marfan syndrome. Mutation analyses have identified more than thirty causative mutations in the FBN1 gene to date.¹⁴⁻²⁶ Concurrently, biochemical analyses have highlighted variations between patient cells with respect to synthesis, secretion, and deposition of fibrillin.^{27,28} However, there are still no satisfactory molecular explanations for how FBN1 mutations influence microfibril assembly and functionality, and generate the disease phenotype.

We have previously used a combination of biochemical and ultrastructural approaches to investigate how different fibrillin mutations influence microfibril formation and organisation.^{29,30} We have shown a range of microfibrillar abnormalities in a panel of Marfan patient cell lines and highlighted differences in fibrillin expression and aggregation between Marfan lines. In this study, we report the results of a similar strategy aimed at gaining insights into why certain fibrillin defects are particularly deleterious within the ocular system and predispose to lens dislocation. Using tissues and cells from two affected members of a three generation Marfan family, we have shown normal secretion and assembly of fibrillin but identified structurally and functionally abnormal microfibrils. In this case, the fibrillin defect which manifests at the macromolecular level clearly underlies their ocular symptoms.

School of Biological Sciences, Stopford Building 2.205, University of Manchester, Oxford Road, Manchester M13 9PT, UK
C M Kielty
J C A Shuttleworth
J E Phillips

Institute of Medical Genetics, University Hospital of South Wales, Cardiff CF4 4XW, UK
S J Davies

Department of Pathological Sciences, University of Manchester, Manchester M13 9PT, UK
C J P Jones

Royal Eye Hospital, Manchester M13 9WH, UK
S J Charles

Correspondence to: Dr Kielty.

Received 13 June 1994
Revised version accepted for publication 1 September 1994

Mutations in the FBN1 gene that encodes the glycoprotein fibrillin are responsible for Marfan syndrome, an autosomal dominant connective tissue disorder that affects cardiovascular and musculoskeletal systems and the eye.¹⁻³ Marfan syndrome has a high penetrance, but is characterised by strikingly heterogeneous inter- and intrafamilial phenotypes.³ In general, Marfan patients manifest some degree of cardiovascular, skeletal, and ocular involvement. However, in some cases, ocular signs, including bilateral ectopia lentis, myopia, and retinal detachment clearly predominate as the major clinical feature.

Material and methods

CLINICAL HISTORY

Ocular tissues and dermal fibroblasts were obtained from two affected cousins (ME and

KC) of a three generation Marfan family. Both patients were tall with arachnodactyly and bilateral ectopia lentis, but valvular function and aortic root dimensions were normal by echo-

Figure 1 Ultrastructural analysis of ciliary zonules from patient ME and control. Transmission electron micrographs of ciliary zonules from patient ME (B,C) and control (A, D). (A) shows a control zonule in longitudinal section. (B) shows a comparable section of patient zonule. (C and D) are cross sections through patient and control ciliary zonules. Rotary shadowing electron micrographs of microfibrils isolated from zonules of patient ME (E) and control (F). Bars = 100 nm.

cardiography. A vitreolensectomy was performed for subluxed crystalline lens in patient ME. At surgery, lens capsule together with attached zonules were obtained for examination together with samples of anterior vitreous.

TRANSMISSION ELECTRON MICROSCOPY

Ciliary zonules and lens capsule removed at vitreolensectomy from ME were fixed with 3% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer (pH 7.4) for four hours at room temperature, and washed three times over 24 hours in 0.1 mol/l sodium cacodylate buffer (pH 7.4) containing 3 mmol/l CaCl_2 . Tissues were then carefully trimmed under a dissecting microscope before postfixation in 1% (w/v) os-

mium tetroxide in 0.5 mol/l sodium cacodylate at 4°C for one hour, and briefly rinsed in buffer before dehydration in an ascending alcohol series. After incubation in two changes of polypropylene oxide, the tissue was infiltrated with TAAB epoxy resin (TAAB Laboratories Equipment Ltd, Aldermaston, UK) and placed in flat embedding moulds for polymerisation at 60°C for 72 hours. Sections 0.5 µmol/l thick were cut on a Reichert OMUIII ultramicrotome and stained with 1% toluidine blue in 1% borax; appropriate areas were then selected for ultrathin sectioning. Pale gold (70 nm) sections were mounted on copper grids and contrasted with uranyl acetate and lead citrate before examination in a Philips EM 301 electron microscope at an accelerating voltage of 60 kV. Control tissues were obtained from

Figure 2 Rotary shadowing electron micrographs of microfibrils isolated from patient dermal fibroblast cell layers. ME and KC cell lines elaborated abundant microfibrils. The predominant abnormality was irregular periodicity, with extended and contracted regions recurring within the same microfibrils (C, E). In some fields, short microfibrillar arrays with a frayed appearance were also present (D, F). (A and B) microfibrils isolated from normal dermal fibroblasts; (C and D), microfibrils isolated from KC cultures; (E and F), microfibrils isolated from ME cultures. Bars = 100 nm.

the Eye Bank at the Manchester Royal Eye Hospital.

ROTARY SHADOWING ELECTRON MICROSCOPY
Tissue and cell layer preparations were visualised for their microfibril content by rotary shadowing electron microscopy using a modification of the mica sandwich technique.⁶ Samples visualised were void volume fractions of cell layer extracts (see below), ciliary zonules (from patient ME) taken up and diluted in 0.5 mol/l Tris/HCl, pH 7.4 containing 0.4 mol/l NaCl, 2 mmol/l phenylmethane-sulphonyl fluoride (PMSF), and 5 mmol/l N-ethyl maleimide (NEM), and vitreous (from patients ME and KC) either neat or diluted 1:1 in the same buffer.

CELLS AND CELL CULTURE
Dermal fibroblast cultures were established by explant from skin biopsies obtained from ME and KC, with the informed consent of both patients. Cells were routinely maintained in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum, penicillin

(400 U/ml), streptomycin (50 mg/ml), and glutamine (200 mg/ml). Confluent cells were labelled with [³⁵S] TranSlabel in medium containing 0.5% fetal calf serum. (TRANS³⁵-LABEL metabolic labelling reagent was supplied by ICN Biomedicals Ltd, Thame, Oxon, UK. This reagent is derived from [³⁵S] *E coli* hydrolysate and contains 70% L-methionine [³⁵S] and 20% L-cysteine [³⁵S]). Proteinase inhibitors (2mmol/l PMSF and 5 mmol/l NEM) were added after 16 hours. Cell layers were solubilised in 0.05 mol/l Tris/HCl, pH 7.4 containing 0.4 mol/l NaCl and 1% (v/v) Nonidet P40 (NNT buffer). Fibrillin was immunoprecipitated from medium and cell layer compartments as previously described.^{7,30} 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 1:1 (v/v) solution of gelatine-Sepharose. Samples were then incubated for one hour at 20°C with a 1:100 dilution of a polyclonal fibrillin antiserum^{7,30} before the addition of 60 µl of a 1:1 (v/v) solution of protein A-Sepharose in NNT buffer.

For microfibril extractions, cells were maintained at post-confluence for up to three weeks. Cell layers were washed in 0.05 mol/l Tris/HCl, pH 7.4 containing 0.4 mol/l NaCl and 0.005 mol/l CaCl₂, then incubated for two hours at 20°C with 0.1 mg/ml bacterial collagenase (type 1A), 2 mmol/l PMSF and 5 mmol/l NEM. Soluble extracts were clarified by centrifugation for 15 minutes at 7500 g on a bench microfuge before size fractionation by gel filtration chromatography on Sepharose CL-2B.⁷

Figure 3 Electrophoretic analysis of fibrillin immunoprecipitated from medium and cell layer extracts of ME dermal fibroblasts. Cells were continuously labelled with [³⁵S] TranSlabel for 16 or 72 hours, or pulse labelled for 30 minutes and chased for two hours and 16 hours. Samples were analysed by SDS-PAGE on 8% gels under non-reducing conditions and by fluorography. The electrophoretic mobilities of molecular weight markers catalase (*M_r* 232 000) and α2-macroglobulin (*M_r* 190 000) are indicated. Tracks 1, 3, 5, and 7, fibrillin immunoprecipitated from medium (M); tracks 2, 4, 6, and 8 fibrillin immunoprecipitated from cell layer extracts (C). When cells were continuously labelled for 16 or 72 hours there was evidence for several fibrillin immunoreactive components in medium (*M_s* 300 000, 270 000, higher *M_r* aggregates and lower *M_r* components; tracks 1 and 3). Failure to detect labelled fibrillin in the cell layer extracts suggests that high *M_r* microfibrillar assemblies may be too large for effective immunoprecipitation (tracks 2 and 4). In the pulse chase experiment, after two hours secreted fibrillin (*M_r* 300 000) was present as a doublet in medium after two hour chase (track 5) and in cell layers (track 6). After 16 hours chase, there was evidence for aggregation of fibrillin in medium (track 7), but again no detectable labelled fibrillin was immunoprecipitated from the cell layer extract (track 8).

Results
OCULAR TISSUES
Transmission electron microscopy of ciliary zonules from patient ME showed the presence of abundant loose microfibril bundles (fig 1). In some fields, the microfibril bundles were markedly disrupted in comparison with those observed in the control tissue (fig 1A,B), and the patient microfibrils had apparently fragmented in places. In cross section, the organisation of some of these bundles appeared similar to the unaffected control (fig 1C,D).
When ciliary zonules from ME and a control were visualised by rotary shadowing

Distribution of counts incorporated into newly synthesised fibrillin by KC and ME cell lines

Cell line	% total incorporated counts in fibrillin	Distribution of fibrillin (%) in	
		Medium	Cell layer
KC	0.118	39	61
ME	0.109	31	69
Control	0.142	36	64

Cells were labelled for 16 hours with [³⁵S] TranSlabel. Fibrillin was immunoprecipitated from medium and cell layer extracts of normal fibroblast culture and KC and ME cell lines. The amount of distribution of newly synthesised fibrillin was compared by determining the percentage of total immunoprecipitated counts in medium and cell layer extracts. The figures are averaged from two duplicate experiments.

electron microscopy, it became apparent that extensive fragmentation had occurred in the patient sample (fig 1E,F). Very few intact microfibrils were observed, but there was evidence of numerous dissociated beaded domains. Visualisation of vitreous from ME and KC showed that, while fibrillin microfibrils were relatively scarce in this tissue, those present were also clearly disrupted (not shown).

CELL CULTURES

Dermal fibroblast cultures were established from both ME and KC in order to investigate these microfibrillar abnormalities further. Examination by rotary shadowing electron microscopy of high relative molecular mass material solubilised from postconfluent cell layers indicated that extensive and abundant microfibrils had been elaborated by control, ME, and KC cultures (fig 2). The microfibrils present in control cultures were similar to those previously isolated from tissues and cells (fig 2A, B).⁶⁷ In marked contrast, microfibrils extracted from ME and KC cell layers were abnormal in several morphological respects (fig 2C-F). In both cases, microfibrillar periodicity was markedly irregular along the microfibrils with areas of highly extended periodicity juxtaposed to contracted regions. In some cases, microfibrillar integrity had clearly been compromised within extended regions. While short microfibrillar arrays from both ME and KC both exhibited "fraying" in interbeaded domains, this was particularly apparent in the case of ME microfibrils. Furthermore, regions of KC microfibrils appeared poorly organised.

The expression and deposition of fibrillin in ME and KC dermal fibroblast cultures was investigated after 16 hours or pulse chase metabolic labelling and immunoprecipitation of fibrillin from medium and cell layer fractions. De novo fibrillin synthesis was expressed as total counts incorporated into fibrillin (table). The two lines synthesised comparable levels of fibrillin and in both cases the majority of newly synthesised fibrillin was deposited in the cell layer. Pulse chase experiments showed that fibrillin was present as a M_r 300 000 component which resolved as a doublet (fig 3). With time, some of this component became incorporated into higher M_r aggregates. Interestingly, no monomers were detected in the cell layer at longer chase intervals. Continuous labelling highlighted a number of lower M_r fibrillin immunoreactive bands in the medium. The presence of these bands suggests that a portion of the newly synthesised fibrillin is catabolised and does not contribute to the formation of stable fibrillin aggregates.

Discussion

The relationship between defined mutations in FBN1, fibrillin defects, and clinical phenotype remains largely obscure despite the documentation of more than thirty mutations to

date and the demonstration of a range of defects in the expression and assembly of fibrillin.^{14-26 29 30} The complexity of the disease is highlighted by the strikingly heterogeneous inter- and intrafamilial phenotypes, and to date no molecular explanation for this phenomenon has emerged. In this study, we have investigated the relationship between defined microfibrillar abnormalities and clinical symptoms in a family whose affected members consistently manifest lens dislocation and skeletal symptoms, but who have no cardiovascular involvement.

Direct ultrastructural examination of patient ciliary zonules confirmed the presence of abundant loose microfibril bundles, and provided evidence for disrupted zonule organisation and fragmentation of microfibrils. When patient ciliary zonules and vitreous were examined by rotary shadowing electron microscopy, it was immediately apparent that the microfibrils were comprehensively fragmented. In order to investigate these abnormal microfibrillar assemblies further, we carried out a biochemical and ultrastructural analysis of fibrillin expression and assembly by patient dermal fibroblasts. These studies established that the pattern of fibrillin secretion and deposition was normal but highlighted that not all the newly synthesised fibrillin was effectively incorporated into high M_r aggregates. Furthermore, the presence of low M_r fibrillin immunoreactive components was suggestive of increased turnover in medium and cell layers. The resolution of fibrillin as a doublet has previously been reported for several other cell lines and taken as indicative of a putative processing event.^{27 31} The ultrastructural observation that abundant microfibrils had been laid down by both cell lines confirmed that ordered assembly of fibrillin had occurred. However, a striking abnormal feature of the microfibrils elaborated by both cell lines was their highly irregular periodicity and evidence for substantial fragmentation within extended regions.

Our results show conclusively that fibrillin microfibrils in these tissues are structurally and functionally abnormal. In view of the exclusively microfibrillar composition of the ciliary zonules, it appears likely that, in these patients, the microfibrils are unable to sustain their role in the dynamic suspension of the lens. In Marfan syndrome it is thought that ectopia lentis results from laxity of the zonular fibres since a good zonular complement is often observed in the area of dislocation.³² Interestingly, ultrastructural analyses have suggested that normal fibrillin microfibrils possess the capacity to extend and retract.⁸ In this study, we have shown markedly variable periodicity within isolated microfibrils suggesting that the normal molecular mechanism for sustaining microfibrillar periodicity is compromised in these patients.

Essentially normal function of the elastic tissues of these patients suggests that the capacity of their microfibrils to interact with elastin has not been affected by the causative mutation. It is interesting to speculate that

the preferential expression of a second fibrillin locus (FBN2) in elastic tissues may in fact compensate for defective FBN1 in these patients. The role of fibrillin in bone, and the influence of microfibrillar abnormalities on the skeletal systems of these patients remain to be defined.

This is the first direct correlation of microfibrillar abnormalities manifest by patient cell cultures and tissues. Future studies will be directed to the association of defined mutations with ultrastructural abnormalities.

This work was supported by the Medical Research Council and the Arthritis and Rheumatism Council. The authors thank Professor D McLeod for his help in this project.

- 1 Lee B, Godfrey M, Vitale E, *et al.* Linkage of Marfan syndrome and a phenotypically related disorder to two different fibrillin genes. *Nature* 1991;352:330-4.
- 2 Tsiopoulos P, Del Mastro R, Sarfarazi M, *et al.* Linkage analysis demonstrates that Marfan syndrome, dominant ectopia lentis and congenital contractural arachnodactyly are linked to the fibrillin genes on chromosomes 15 and 5. *N Engl J Med* 1992;326:905-9.
- 3 Pyeritz RE. The Marfan syndrome. In: Royce PM, Steinmann B, eds. *Connective tissue and its heritable disorders*. New York: Wiley-Liss, 1993:437-67.
- 4 Sakai LY, Kenne DR, Engvall E. Fibrillin, a new 350-kD glycoprotein, is a component of extracellular microfibrils. *J Cell Biol* 1986;103:4299-509.
- 5 Sakai LY, Keene DR, Glanville RW, Bachinger HP. Purification and partial characterisation of fibrillin, a cysteine-rich structural component of connective tissue microfibrils. *J Biol Chem* 1991;266:14763-70.
- 6 Kielty CM, Cummings C, Whittaker SP, Shuttleworth CA, Grant ME. Isolation and ultrastructural analysis of microfibrillar structures from foetal bovine elastic tissues. *J Cell Sci* 1991;99:797-807.
- 7 Kielty CM, Berry L, Whittaker SP, Grant ME, Shuttleworth CA. Microfibrillar assemblies of foetal bovine skin. *Matrix* 1993;13:103-12.
- 8 Keene DR, Maddox BK, Kuo HJ, Sakai LY, Glanville RW. Extraction of extendable beaded structures and their identification as fibrillin-containing extracellular microfibrils. *J Histochem Cytochem* 1991;39:441-9.
- 9 Wallace RN, Streeten BW, Hanna RB. Rotary shadowing of elastic system microfibrils in the ocular zonule, vitreous and ligamentum nuchae. *Curr Eye Res* 1991;10:99-109.
- 10 Wright DW, Mayne R. Vitreous humor of chicken contains two fibrillar systems: an analysis of their structure. *J Ultrastruct Molec Struct Res* 1988;100:224-34.
- 11 Wright DW, McDaniels CN, Swasdison S, Accavitti MA, Mayne PM, Mayne R. Immunization with undenatured bovine zonular fibrils results in monoclonal antibodies to fibrillin. *Matrix Biol* 1994;14: 41-9.
- 12 Fleischmajer R, Contard P, Schwartz E, MacDonald ED, Jacobs L, Sakai LY. Elastin-associated microfibrils (10nm) in a three-dimensional fibroblast culture. *J Invest Dermatol* 1991;97:639-43.
- 13 Zhang H, Apfelroth SD, Hu W, *et al.* Structure and expression of fibrillin-2, a novel microfibrillar component preferentially located in elastic matrices. *J Cell Biol* 1994;124:855-63.
- 14 Dietz HC, Cutting GR, Pyeritz RE, *et al.* Marfan syndrome caused by a recurrent de novo missense mutation in the fibrillin gene. *Nature* 1991;352:337-9.
- 15 Dietz HC, Saraiva JM, Pyeritz RE, Cutting GR, Francomano CA. Clustering of fibrillin (FBN1) missense mutations in Marfan syndrome patients at cysteine residues in EGF-like domains. *Hum Mutat* 1992;1: 366-74.
- 16 Dietz HC, Pyeritz RE, Puffenberger EG, *et al.* Marfan phenotype variability in a family segregating a missense mutation in the epidermal growth factor-like motif of the fibrillin gene. *J Clin Invest* 1992;89:1674-80.
- 17 Dietz HC, Valle D, Francomano CA, Kendzior RJ, Pyeritz RE, Cutting GR. The skipping of constitutive exons in vivo induced by nonsense mutations. *Science* 1993;259: 680-3.
- 18 Dietz HC, McIntosh I, Sakai LY, *et al.* Four novel FBN1 mutations: significance for mutant transcript level and EGF-like domain calcium binding in the pathogenesis of Marfan syndrome. *Genomics* 1993;17:468-75.
- 19 Kainulainen K, Peltonen L. Marfan syndrome: molecular pathogenesis. *Adv Genome Biol* 1994;2:113-33.
- 20 Kainulainen K, Sakai LY, Child AH, *et al.* Two mutations in Marfan syndrome resulting in truncated fibrillin polypeptides. *Proc Natl Acad Sci USA* 1992;89:5917-21.
- 21 Kainulainen K, Karttunen K, Puhakka L, Sakai LY, Peltonen L. Mutations in the fibrillin gene responsible for dominant ectopia lentis and neonatal Marfan syndrome. *Nature Genet* 1994;6:64-9.
- 22 Godfrey M, Vandemark N, Wang M, *et al.* Prenatal diagnosis and a donor splice site mutation in fibrillin in a family with Marfan syndrome. *Am J Hum Genet* 1993;49:662-7.
- 23 Hewitt DR, Lynch JR, Smith R, Sykes B. A novel fibrillin mutation in the Marfan syndrome which could disrupt calcium binding of the epidermal growth factor-like module. *Hum Molec Genet* 1993;2:475-7.
- 24 Tynan K, Comeau K, Pearson M, *et al.* Mutation screening of complete fibrillin-1 coding sequence: report of five new mutations, including two in 8-cysteine domains. *Hum Molec Genet* 1993;2:1813-21.
- 25 Hayward C, Porteous MEM, Brock DJH. Identification of a novel nonsense mutation in the fibrillin gene (FBN1) using nonisotopic techniques. *Hum Mutat* 1994; 3:159-62.
- 26 Milewicz DM, Duvic M. Severe neonatal Marfan syndrome resulting from a de novo 3-bp insertion into the fibrillin gene on chromosome 15. *Am J Hum Genet* 1994;54: 447-53.
- 27 McGookey Milewicz D, Pyeritz RE, Crawford ES, Byers PH. Marfan syndrome: defective synthesis, secretion and extracellular matrix formation of fibrillin by cultured dermal fibroblasts. *J Clin Invest* 1992;89:79-86.
- 28 Aoyama T, Tynan K, Dietz HC, Francke U, Furthmayr H. Missense mutations impair intracellular processing of fibrillin and microfibril assembly in Marfan syndrome. *Hum Molec Genet* 1994;2:2135-40.
- 29 Kielty CM, Shuttleworth CA. Abnormal fibrillin assembly by dermal fibroblasts from two patients with Marfan syndrome. *J Cell Biol* 1994;124:997-1004.
- 30 Kielty CM, Phillips JE, Child AH, Pope FM, Shuttleworth CA. Fibrillin secretion and microfibril assembly by Marfan dermal fibroblasts. *Matrix Biol* 1994;14:191-9.
- 31 Raghunath M, Superti-Furga A, Godfrey M, Steinmann B. Decreased deposition of fibrillin and decorin in neonatal Marfan syndrome fibroblasts. *Hum Genet* 1993; 90:5411-15.
- 32 Nelson LB, Maumane IH. Ectopia lentis. *Surv Ophthalmol* 1986;27:143-60.