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Collagen VI Glycine Mutations: Perturbed Assembly and a Spectrum of Clinical Severity

Rishika A. Pace, BSc(Hons)¹, Rachel A. Peat, PhD², Naomi L. Baker, PhD¹, Laura Zamurs, PhD¹, Matthias Mörgelin, PhD³, Melita Irving, MBBS¹, Naomi E. Adams, BSc(Hons)¹, John F. Bateman, PhD¹, David Mowat, MBBS⁴, Nicholas J. C. Smith, MBBS⁵, Phillipa J. Lamont, MBBS, PhD⁶, Steven A. Moore, MD, PhD^{7,8}, Katherine D. Mathews, MD^{9,10}, Kathryn N. North, MBBS, MD², and Shireen R. Lamandé, PhD¹

¹Murdoch Childrens Research Institute and Department of Paediatrics, University of Melbourne, Royal Children's Hospital, Victoria

²Institute for Neuromuscular Research, Children's Hospital at Westmead and Discipline of Paediatrics and Child Health, University of Sydney, New South Wales, Australia

³Department of Clinical Sciences, Lund University, Lund, Sweden

⁴Department of Medical Genetics New South Wales

⁵Department of Neurology, Sydney Children's Hospital, New South Wales

⁶Neurogenetics Unit, Department of Neurology, Royal Perth Hospital, Perth, Australia

⁷Department of Pathology, University of Iowa

⁸Iowa Wellstone Muscular Dystrophy Cooperative Research Center

⁹Department of Pediatrics, University of Iowa

¹⁰Iowa Wellstone Muscular Dystrophy Cooperative Research Center, Iowa City, IA.

Abstract

Objective—The collagen VI muscular dystrophies, Bethlem myopathy and Ullrich congenital muscular dystrophy, form a continuum of clinical phenotypes. Glycine mutations in the triple helix have been identified in both Bethlem and Ullrich congenital muscular dystrophy, but it is not known why they cause these different phenotypes.

Methods—We studied eight new patients who presented with a spectrum of clinical severity, screened the three collagen VI messenger RNA for mutations, and examined collagen VI biosynthesis and the assembly pathway.

Results—All eight patients had heterozygous glycine mutations toward the N-terminal end of the triple helix. The mutations produced two assembly phenotypes. In the first patient group, collagen VI dimers accumulated in the cell but not the medium, microfibril formation in the medium was moderately reduced, and the amount of collagen VI in the extracellular matrix was not significantly altered. The second group had more severe assembly defects: some secreted collagen VI tetramers

were not disulfide bonded, microfibril formation in the medium was severely compromised, and collagen VI in the extracellular matrix was reduced.

Interpretation—These data indicate that collagen VI glycine mutations impair the assembly pathway in different ways and disease severity correlates with the assembly abnormality. In mildly affected patients, normal amounts of collagen VI were deposited in the fibroblast matrix, whereas in patients with moderate-to-severe disability, assembly defects led to a reduced collagen VI fibroblast matrix. This study thus provides an explanation for how different glycine mutations produce a spectrum of clinical severity.

The collagen VI muscular dystrophies include Bethlem myopathy (MIM 158810) and Ullrich congenital muscular dystrophy (UCMD; MIM 254090). The clinical features of these disorders have been recently reviewed.¹ In brief, Bethlem myopathy was first described as a mild dominantly inherited disorder with the onset of symptoms within the first or second decade of life.² Joint contractures are a hallmark of the disorder, and most patients have flexion contractures of the fingers, wrists, elbows, and ankles. The disorder is slowly progressive, and the majority of patients older than 50 years require aids for ambulation.³ In contrast, muscle weakness in UCMD is profound, onset is early or congenital, and patients either never achieve independent ambulation or walk for only a few years.¹ Patients have proximal joint contractures and striking distal hyperlaxity. Other common features include congenital hip dislocation, protruding calcanei, follicular hyperkeratosis, a round face, prominent ears, soft velvety skin, and abnormal scarring. UCMD was initially described as a recessive condition, and the first mutations described were recessive⁴; however, it was subsequently shown that dominant mutations can also cause the severe UCMD phenotype.^{5,6} With the identification of increasing numbers of dominant and recessive mutations, and description of the resulting clinical phenotypes, it has become clear that the classically described Bethlem myopathy and UCMD phenotypes can no longer be considered distinct entities but opposite ends of a spectrum of disorders.¹

Collagen VI is an extracellular matrix protein with a broad tissue distribution.⁷ In skeletal muscle it is found closely associated with the basement membrane and is thought to link the basement membrane to the surrounding extracellular matrix. The three protein chains of collagen VI, $\alpha 1(VI)$, $\alpha 2(VI)$, and $\alpha 3(VI)$, are encoded by *COL6A1*, *COL6A2*, and *COL6A3*, respectively, and mutations in all three genes underlie the collagen VI muscular dystrophies.¹ Collagen VI has a complex assembly pathway. Within cells, the three chains associate initially via the C-terminal globular domains, and the triple helix folds from the C to N terminus to form the collagen VI monomer.⁸⁻¹⁰ Dimers then form by antiparallel staggered alignment of the monomers and are stabilized by disulfide bonds. Lateral association of dimers and further disulfide bond formation results in tetramers, the secreted form of collagen VI. Outside the cell, collagen VI tetramers align end to end into the characteristic beaded microfibrils.⁷

Although more than 60 dominant and recessive collagen VI mutations have been identified,¹ detailed analyses of the effects of the mutations on assembly of the protein have been conducted on only a small number of patients^{5,6,9,11}; thus, our understanding of the relationship between the type of mutation and the clinical presentation is limited. One class of mutations that has been identified in patients with both Bethlem myopathy and UCMD is glycine substitutions that interrupt the repeating Gly-X-Y sequence motif of the triple-helical domain.¹²⁻¹⁹ To begin to understand the relationship between genotype and phenotype for this class of mutations, we have studied eight new patients who have dominant glycine mutations in the collagen VI triple helix and presented with a spectrum of clinical phenotypes from mild Bethlem myopathy to severe UCMD.

Subjects and Methods

Patient Samples

Dermal fibroblast cultures were established from eight patients with suspected collagen VI muscular dystrophies, and genomic DNA was extracted from peripheral blood samples obtained from their parents. Muscle biopsies were available from seven of the patients. Samples were collected after informed consent and approval of the Royal Children's Hospital Ethics in Human Research Committee and the University of Iowa Human Subjects Committee were obtained.

Muscle Biopsy Staining

Frozen sections (8 μ m), cut from muscle biopsies using a CM1900 Cryostat (Leica, Deerfield, IL), were mounted onto Superfrost Plus slides (Menzel-Glaser, Braunschweig, Germany) and immunostained with collagen VI and perlecan antibodies, as described previously.⁶ Images were obtained using an Olympus BX50F4 (Olympus, Tokyo, Japan) microscope at 40 $\times$ magnification.

Mutational Analysis by Reverse Transcriptase Polymerase Chain Reaction, Genomic Polymerase Chain Reaction, and Sequencing

Total RNA was isolated from confluent fibroblasts using RNeasy (Qiagen, Chatsworth, CA) and reverse transcribed using a GeneAmp RNA Polymerase Chain Reaction (PCR) Kit (Applied Biosystems, Foster City, CA). The resulting complementary DNA was used as a template for PCR amplification of the entire coding regions of the α 1(VI) and α 2(VI) messenger RNA (mRNA) (7 primer pairs for each), and the region of the α 3(VI) mRNA coding for protein domains N3-C5 (12 primer pairs). Reverse transcriptase (RT) PCR and sequencing conditions were described previously.⁶ The heterozygous mutations detected in RT-PCR products were confirmed by genomic PCR and sequencing. When available, genomic DNA from the parents was also PCR amplified and sequenced.

Collagen VI Biosynthetic Labeling and Analysis

Primary dermal fibroblasts were grown to confluence in 10cm² dishes, then incubated overnight in the presence of 0.25mM sodium ascorbate. Biosynthetic labeling with [³⁵S] methionine, immunoprecipitation using an α 3(VI) N1 domain antibody and polyacrylamide, and composite gel electrophoresis conditions were as described previously.⁶ In some experiments, the medium was removed after biosynthetic labeling and clarified by centrifugation; then 0.5ml aliquot was separated by gel filtration chromatography under native conditions on a Superose-6 10/300 GL column (GE Healthcare, Chalfont, St. Giles, UK). The column buffer was 50mM Tris/HCl, pH 7.5, 150mM NaCl; the flow rate was 0.5ml/min, and 0.5ml fractions were collected. Protease inhibitors were added to appropriate fractions, and the collagen VI was immunoprecipitated as before.⁶

Immunostaining of Fibroblast Extracellular Matrix

Fibroblasts were grown to confluence in four-well chamber glass slides (Becton Dickinson, San Jose, CA) and then supplemented daily for 2 days with 0.25mM sodium ascorbate. Collagen VI in the extracellular matrix was visualized by staining, before fixation, using the collagen VI antibody 3C4, as described previously.⁶

Electron Microscopy

The medium from confluent human fibroblasts that had been incubated overnight with serum-free medium containing 0.25mM sodium ascorbate was collected and prepared for negative

staining electron microscopy, as described previously.⁶ Samples were observed in Jeol 1200EX electron microscope operated at 60kV accelerating voltage.

Results

Patients

The eight patients included in this study presented with clinical features consistent with a diagnosis of a collagen VI muscular dystrophy,^{20,21} including proximal muscle weakness and distal hyperlaxity, normal or mildly increased serum creatine kinase level, and no apparent heart involvement (see Supplementary Table S1). All patients had congenital onset of disease and were thus classified as having UCMD. Seven patients had follicular hyperkeratosis (presenting as a rough skin rash), and five had prominent heels. These features are commonly seen in UCMD patients.¹ Motor disability varied from relatively mild to severe: three patients were unable to run (UCMD Patients 7, 8, and 31); two were capable of walking short distances (UCMD Patients 41 and 9), although UCMD Patient 9 was largely restricted to a wheel chair; one became wheel-chair bound at 5 years of age (UCMD Patient 46); and two never achieved independent ambulation (UCMD Patients 20 and 38). None of the patients had a family history of muscular dystrophy.

Collagen VI Is Abnormally Localized in Skeletal Muscle

Muscle biopsies were available from seven patients and were immunostained with collagen VI and perlecan antibodies. Collagen VI was present in the muscle of all six patients but was no longer colocalized with perlecan at the basement membrane as seen in control subjects (Fig 1). This is a characteristic finding in UCMD^{5,6,18,22-25} and confirms the diagnosis of a collagen VI muscular dystrophy.

All Eight Patients Have Glycine Mutations in the Collagen VI Triple Helix

To identify the underlying collagen VI mutations in these patients, we amplified the entire coding regions of the $\alpha 1$ (VI) and $\alpha 2$ (VI) mRNA and the region of the $\alpha 3$ (VI) mRNA encoding domains N3-C5 by RT-PCR, and directly sequenced. All eight patients had heterozygous single-base changes leading to glycine substitutions toward the N-terminal end of the triple helix: $\alpha 1$ (VI) c.850G>A (p.G284R) in UCMD Patient 20; $\alpha 1$ (VI) c.868G>A (p.G290R) in UCMD Patient 41; $\alpha 1$ (VI) c.887G>T (p.G296V) in UCMD Patient 8; $\alpha 2$ (VI) c.785G>A (p.G262D) in UCMD Patient 31; $\alpha 2$ (VI) c.982G>A (p.G298R) in UCMD Patient 9; $\alpha 3$ (VI) c.6194G>A (p.G2065D) in UCMD Patient 46; $\alpha 3$ (VI) c.6221G>A (p.G2074D) in UCMD Patient 38; and $\alpha 3$ (VI) c.6284G>T (p.G2095V) in UCMD Patient 7 (see Supplementary Table S2). The $\alpha 1$ (VI) p.G284R and p.G290R mutations have been reported previously^{13,18,22}; however, the other six mutations are novel. The presence of the mutations was confirmed in all patients by sequencing genomic DNA PCR products (data not shown). The unaffected parents of UCMD Patients 7, 8, 20, 31, and 38 did not carry the glycine substitution mutations, indicating that they were de novo mutations in the patients (data not shown). DNA was available only from the father of UCMD Patient 9, and he did not carry the mutations. We were not able to obtain DNA from the parents of UCMD Patients 41 and 46. Many additional sequence variations were also present in the RT-PCR products from the eight patients (see Supplementary Table S2). All but six of these changes have been identified previously in unaffected individuals and were thus considered nonpathogenic.^{5,6,13,18,26} Three novel heterozygous changes, $\alpha 2$ (VI) c.225G>A (p.P75P) in UCMD Patient 31, $\alpha 2$ (VI) c.2880 G>A (p.S960S) in UCMD Patient 7, and $\alpha 3$ (VI) c.5619C>T (p.H1873H) in UCMD Patient 38, did not alter the amino acid sequence and did not affect mRNA splicing (data not shown), and were thus considered nonpathogenic. A novel $\alpha 2$ (VI) change, c.2260G>A (p.R867Q) in UCMD Patient 7, was carried by the patient's unaffected father, and $\alpha 3$ (VI) c.5693T>C (p.V1898A) in UCMD Patient 38 was inherited from his unaffected father. These two amino acid changes

were also considered nonpathogenic. The remaining change, $\alpha 3(\text{VI})$ c.9028A>G (p.R3010G) in UCMD Patient 9, was not found in more than 100 control chromosomes. The father of UCMD Patient 9 did not carry the change; however, DNA was not available from the mother. R3010 is conserved in human, dog, and mouse but is a serine residue in chicken and *Xenopus*, and an asparagine in opossum and platypus (data not shown), suggesting that amino acid changes at this position may be tolerated without phenotypic consequences.

Collagen VI Intracellular Assembly Is Compromised by the Triple-Helical Glycine Mutations

To determine the effect of the mutations on collagen VI biosynthesis and assembly, we biosynthetically labeled control and patient fibroblasts with [^{35}S]methionine. Collagen VI in the cell and medium fractions was immunoprecipitated with an antibody made to the $\alpha 3(\text{VI})$ N1 domain,^{6,8} and analyzed under nonreducing conditions on composite agarose-acrylamide gels to visualize collagen VI monomers, dimers, and tetramers. In addition to our seven new patients, we also reanalyzed the collagen VI produced by a Bethlem myopathy cell line harboring a heterozygous $\alpha 1(\text{VI})$ p.G305V mutation.^{9,12} Collagen VI tetramers were the major assembly form in the cell and medium from control subjects (Fig 2). Tetramers were also the predominant form in the Bethlem myopathy cell and medium fractions (see Fig 2, lane 10), confirming our published data demonstrating that the $\alpha 1(\text{VI})$ p.G305V mutation did not prevent disulfide bonding of the tetramers.⁹ In contrast, all eight newly identified mutations compromised intracellular assembly and disulfide bonding of the tetramers. The patients fell into two groups: two patients, UCMD Patients 7 and 31, had significant amounts of intracellular dimers but the secreted collagen VI was predominantly tetrameric (see Fig 2, lanes 3, 4, 17, and 18); in the other six patients, UCMD Patients 8, 9, 20, 38, 41, and 46, collagen VI dimers were also apparent in the medium (see Fig 2, lanes 6, 8, 14, 22, 26, 30).

Impaired Collagen VI Microfibril Formation

The ability of the secreted collagen VI to assemble end to end to form microfibrils was determined using negative staining electron microscopy to quantitate the relative sizes of the microfibrils in fibroblast culture medium.^{8,9,27} In control fibroblast medium, up to 10 tetramers were present and only around 20% of the “microfibrils” were single tetramers (Fig 3). In comparison, microfibril formation was impaired in the patients with glycine mutations, and again the patients fell into two groups. Patient fibroblasts that had dimers in the medium by composite gel electrophoresis had severely impaired microfibril formation with 70 to 80% of the microfibrils containing only one tetramer (see Fig 3). Microfibril formation was not as severely affected in patients who secreted only tetramers (UCMD Patients 7 and 31); they had fewer single tetramers (30–45%) and more microfibrils containing two to three tetramers than the other patient group (see Fig 3).

Collagen VI deposited into the extracellular matrix of confluent fibroblasts treated with sodium ascorbate for 2 days was visualized by immunostaining to determine whether the impaired microfibril formation in the medium was reflected in the extracellular matrix. In UCMD Patients 7 and 31, the cell lines that secreted only tetramers, the collagen VI matrix was similar to the control subjects (Fig 4). As reported previously, the Bethlem myopathy cells also deposited a similar amount of collagen VI as controls.⁹ In contrast, the matrix was severely reduced in the patients who secreted dimers, UCMD Patients 8, 9, 20, 38, 41, and 46.

Secretion of Nondisulfide-Bonded Tetramers

Correlation between the presence of collagen VI dimers in the medium samples of UCMD Patients 8, 9, 20, 38, 41, and 46 on composite gels and severely reduced microfibril formation and/or matrix deposition led us to examine the assembly state of collagen VI in more detail. The composite agarose-acrylamide gels were run under nonreducing conditions; however, they contained sodium dodecyl sulfate and were thus denaturing. To determine whether collagen

VI was secreted as dimers or had assembled into tetramers that were not disulfide bonded and thus migrated as dimers under denaturing conditions, we separated proteins in the biosynthetically labeled medium from control and UCMD cultures by size exclusion chromatography under native conditions. Collagen VI in the eluted column fractions was immunoprecipitated and analyzed as before on nonreducing composite gels. In the control, collagen VI eluted in fractions 15 to 17 and appeared as tetramers on the gel (approximately 2,000kDa; Fig 5A). Fibronectin dimers (approximately 500kDa), which bind nonspecifically to the protein A-Sepharose in the immunoprecipitations,⁸ was apparent predominantly in fractions 20 to 23. Similarly, in UCMD Patient 8, collagen VI tetramers were eluted in fractions 15 to 17 and fibronectin in fractions 20 to 23 (see Fig 5B). Fraction 15 contained only tetramers. Because this fraction consists of the highest molecular weight material it is likely to contain the collagen VI tetramers that have joined end to end to form microfibrils (see Fig 3). This is supported by the large amount of collagen VI in fraction 15 from control cells (see Fig 5A) where the majority of the tetramers have assembled end to end with at least one other tetramer (see Fig 3). However, UCMD Patient 8 fractions 16 and 17 also contained collagen VI that migrated as dimers on the gel. Dimers were not eluted in later fractions (18–20), indicating that under native conditions they were the same size as tetramers. These data suggest that dimers containing mutant chains are able to assemble into tetramers, but stabilization of the tetramers by disulfide bonding is compromised. Similar size exclusion chromatography elution profiles and composite gel migration results were obtained for UCMD Patients 9, 20, 38, 41, and 46 (see Supplementary Fig S1), indicating that fibroblasts from all these patients secrete some tetramers that are not disulfide bonded. We did not observe collagen VI dimers in the medium from UCMD Patients 8, 9, and 20 by electron microscopy (data not shown), providing additional support for the proposal that collagen VI was secreted as nondisulfide-bonded tetramers.

Discussion

The mutations identified in this study bring the total number of published dominant glycine mutations in the triple-helical domain of collagen VI to 24 (see Supplementary Table S3).¹²⁻¹⁹ All the mutations are in the N-terminal third of the helix (Fig 6). Until now, detailed information on the effects of the mutations on collagen VI assembly has been available for only two Bethlem myopathy mutations, $\alpha 1(VI)$ p.G305V and $\alpha 2(VI)$ p.G271S.⁹ Intracellular assembly and disulfide bonding was apparently normal in these patients; there was a reduction in the size of microfibrils in the medium, but the amount of collagen VI in the extracellular matrix was not significantly reduced.

The eight new patients reported here fell into two groups based on assembly defects. In the first group, when compared with control subjects, collagen VI dimers accumulated in the cell but not the medium, microfibril formation in the medium was moderately reduced, yet the amount of collagen VI in the extracellular matrix was not significantly altered (UCMD Patients 7 and 31). The second group had more severe assembly defects: fibroblasts secreted some collagen VI tetramers that were not disulfide bonded, microfibril formation in the medium was severely compromised, and collagen VI in the extracellular matrix was significantly reduced (UCMD Patients 8, 9, 20, 38, 41, and 46).

An explanation for the effects of these mutations on disulfide bonding of the tetramers lies in their location toward the N-terminal end of the triple helix, in the region of the helix that does not overlap in the antiparallel, staggered dimers (see Fig 6). The $\alpha 3(VI)$ cysteine residue involved in disulfide bonding in the tetramers is near the center of this region (residue 50 of the helix), and disruption of the triple-helical structure by the glycine mutations thus has the potential to interfere with interactions important for tetramer assembly and/or alter the orientation of the cysteine residues and prevent normal disulfide bonding.

To understand why some glycine mutations in this region have no detectable effect on disulfide bonding whereas others result in secretion of nondisulfidebonded tetramers, we compared the relative positions of the mutations (see Fig 6). Although data are available on only 10 different glycine mutations, the 6 mutations that result in secretion of some nondisulfide-bonded tetramers are clustered between residues 28 and 43 of the triple helix, and the 4 mutations that result in secretion of only disulfide-bonded tetramers are either N- or C-terminal of this region. This suggests that interactions important for tetramer formation may involve residues 28 to 43 of the helix; however, identification of further glycine mutations and characterization of their effects on collagen VI assembly are needed to confirm this hypothesis. Severe assembly defects and reduced fibroblast collagen VI matrix have also been reported in UCMD patients with dominant in-frame deletions toward the N-terminal end of the helix.⁶ Two deletions, $\alpha 2(VI)$ helical residues 13 to 39 (encoded by exon 6 and part of exon 7) and $\alpha 3(VI)$ triple helical residues 16 to 33 (encoded by exon 16), partially overlap the critical tetramer assembly region defined by the glycine mutations (see Fig 6). Deletion of triple-helical residues 55 to 63 of the $\alpha 1(VI)$ chain (encoded by exon 12) also results in severe collagen VI assembly defects. These residues are C-terminal of amino acids 28 to 43; however, their deletion will alter the amino acid sequence and the structure of the triplehelix N terminus to the deletion, and this is consistent with the observed severe assembly defects.

During collagen VI microfibril formation, the N-terminal domains of adjacent tetramers are predicted to overlap in the junctional complex, allowing multiple potential interactions with each other, with the C-terminal domains and with the N-terminal region of the triple helix of the adjacent tetramer.²⁸⁻³⁰ Microfibrils are not stabilized by covalent bonds, and our results showing impaired microfibril formation in all the patients examined to date with glycine mutations in the N-terminal region of the triple helix indicate that this region plays a fundamental role in either the initiation or maintenance of microfibril structure. The correlation between secretion of some nondisulfide-bonded tetramers and a severe reduction in microfibril assembly further suggests that correct disulfide bonding of the tetramers is critical for the interactions that allow microfibril formation.

Genotype-phenotype correlations in the collagen VI muscular dystrophies is complicated by significant interfamilial and intrafamilial variation,^{14,16,17,20,21,31-33} the age of patients at assessment, and that there are no clinical features that clearly identify a patient as having Bethlem myopathy or UCMD. It is now increasingly recognized that the collagen VI muscular dystrophies form a continuum of clinical phenotypes.^{1,17,18} Although Bethlem myopathy as first described² is readily distinguished from the most severe cases of UCMD,^{34,35} patients that fall toward the center of the disease spectrum are more difficult to classify. It is likely that different studies have used different clinical criteria to classify their patients, adding further complexity to attempts to draw genotype-phenotype correlations. Two studies have graded UCMD patients according to ambulatory impairment: severe UCMD patients never walked independently, moderate UCMD patients lost ambulation before 10 years, and mild UCMD patients remained ambulatory after 10 years of age.^{15,22} In this classification system, it is difficult to know what distinguishes mild UCMD patients from Bethlem myopathy. The presence of distal hyperlaxity was suggested as one feature.¹⁵ However, although distal hyperlaxity is common in UCMD, it is also often seen in Bethlem myopathy.^{1,3,21,31} Another feature proposed to differentiate UCMD from Bethlem myopathy is the degree of motor disability in the first decade of life; if motor impairment was significant, then a diagnosis of UCMD was suggested. Another study subdivided patients into four groups: mild Bethlem myopathy, Bethlem myopathy, severe Bethlem myopathy, and UCMD.¹³ However, the clinical features defining each group were not explained. There is an urgent need to develop a rational clinical classification system for the collagen VI muscular dystrophies that will aid clinicians and researchers in drawing genotype-phenotype correlations and, ultimately, provide patients with accurate information about the expected clinical course of their disease.

In this study, all eight patients had congenital onset of disease and were thus classified as having UCMD. However, the patients presented with a spectrum of clinical severity. UCMD Patient 31 has mild disease and, at 14 years old, fits within the classic Bethlem myopathy phenotype.² Although he presented in the newborn period with hip dysplasia that resolved with bracing, he can achieve a slow run and recently completed a 20km wilderness walk. UCMD Patient 7 also has relatively mild disease but is more severely affected than UCMD Patient 31; UCMD Patient 7 has a normal walking gait with no major contractures, is able to arise from the floor in 3 seconds, but is unable to run. UCMD Patients 8 and 41 have moderately severe disease; UCMD Patient 41 is still able to walk short distances at 13 years old, UCMD Patient 8 has a slow and awkward walking gait and takes more than 20 seconds to rise from a supine to a standing position. The most severely affected patients are UCMD Patients 9, 46, 20, and 38. UCMD Patient 9 is largely restricted to a wheelchair at age 12, UCMD Patient 46 became wheelchair bound at 5 years, and UCMD Patients 20 and 38 never achieved independent ambulation. It is significant that the two mildly affected patients, UCMD Patients 31 and 7, both have mild collagen VI assembly defects, where only disulfide bonded tetramers are secreted, microfibril formation is moderately reduced, and the fibroblast matrix contains normal amounts of collagen VI. The other six patients, with moderate-to-severe UCMD, have severe assembly defects that lead to a significantly reduced amount of collagen VI in the extracellular matrix of fibroblasts. Only two of the mutations in our eight patients have been reported previously, $\alpha 1(\text{VI})$ p.G290R^{13,15,18} and $\alpha 1(\text{VI})$ p.G284R.^{13,15,18} The $\alpha 1(\text{VI})$ p.G290R mutation was found in two patients with severe Bethlem myopathy and two with UCMD,¹³ as well as one UCMD patient who walks with support as a teenager and whose fibroblast matrix contains reduced amounts of collagen VI.¹⁵ Seven other patients have the $\alpha 1(\text{VI})$ p.G284R mutation; all of them have severe disease.^{13,15,18} One patient was shown to have a reduced collagen VI extracellular matrix.¹⁵ These data fit well with both the assembly defects and clinical phenotypes of UCMD Patients 41 and 20. Our data on the consequences of triple-helical glycine mutations on the collagen VI assembly pathway showing that mildly affected patients have mild assembly defects whereas severe assembly defects result in moderate-to-severe disability thus provides, for the first time, an explanation for how different dominant glycine mutations lead to a spectrum of clinical severity from mild Bethlem myopathy to severe UCMD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.

Immunohistochemical staining of collagen VI in muscle from six patients. Frozen sections of muscle were taken from patient and age-matched control biopsies, and stained with antibodies to collagen VI (red) and perlecan (green). Overlays of the collagen VI and perlecan images show colocalization of collagen VI with perlecan in control biopsies. In patients, collagen VI is no longer closely associated with perlecan in the basement membrane. UCMD = Ullrich congenital muscular dystrophy.

Fig 2.

Electrophoretic analysis of collagen VI produced by patient fibroblasts. Control and patient fibroblasts (U7, U8, U9, U20, U31, U38, U41) were biosynthetically labeled overnight with [^{35}S]methionine, and the collagen VI in the cell (C) and medium (M) fractions immunoprecipitated with an $\alpha 3(\text{VI})$ chain antibody. Fibroblasts from a Bethlem myopathy patient with a previously defined $\alpha 1(\text{VI})$ p.G305V mutation (BM) were labeled for comparison. Samples were analyzed without reduction on composite agarose-acrylamide gels. Collagen VI dimers and tetramers are indicated on the right. When compared with the controls, collagen VI dimers accumulated in the cell fractions from Ullrich congenital muscular dystrophy (UCMD) Patients 7 and 31 but not the medium. In the remaining patients, some of the secreted collagen VI migrated as dimers on these denaturing gels.

Fig 3.

Quantitative analysis of collagen VI tetramer-tetramer association in patients with triple-helical glycine mutations. Collagen VI secreted into the medium of control (open squares) and Ullrich congenital muscular dystrophy (UCMD) Patients 7 (large solid squares), 8 (circles), 9 (triangles), 20 (diamonds), and 31 (small solid squares) was visualized by negative staining electron microscopy, and the ability of the tetramers to associate end to end was quantitated. The occurrence of microfibrils containing 1 to 10 tetramers is shown as a percentage of the total number of microfibrils.

Fig 4.

Collagen VI in the fibroblast extracellular matrix. Fibroblasts from control subjects, Ullrich congenital muscular dystrophy (UCMD) patients, and a Bethlem myopathy patient with an $\alpha 1$ (VI) p.G305V mutation (BM) were grown for 2 days after confluence in the presence of sodium ascorbate and collagen VI in the extracellular matrix detected using a collagen VI antibody. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Patient images are shown with a control from the same experiment. UCMD Patients 7 and 31 and the Bethlem myopathy fibroblast matrices contained a similar amount of collagen VI to the controls; however, the collagen VI matrix was significantly reduced in the other five UCMD patient cells lines.

Fig 5.

Ullrich congenital muscular dystrophy (UCMD) fibroblasts secrete nondisulfide-bonded tetramers. Control (A) and UCMD8 (B) fibroblasts were biosynthetically labeled overnight with [³⁵S]methionine and aliquots of the medium separated under native conditions on a Superose 6 10/300 GL column. Collagen VI in the eluted fractions was immunoprecipitated and analyzed under nonreducing conditions on composite agarose-acrylamide gels. Collagen VI tetramers eluted in fractions 15 to 17 and fibronectin dimers (FN₂), which bind nonspecifically to protein A-Sepharose, eluted in fractions 20 to 23. Collagen VI migrating as dimers is apparent in UCMD fractions 16 and 17; however, dimers are not eluted in later fractions, indicating that under native conditions they are the same size as tetramers.

Fig 6.

Dominant collagen VI triple-helical glycine mutations. Schematic showing the structure of collagen VI monomers, dimers, and tetramers. The sequence of the N-terminal end of the triple helix of $\alpha 1(VI)$, $\alpha 2(VI)$, and $\alpha 3(VI)$, where all the dominant collagen VI glycine mutations are located, is shown and expanded below the dimer. The amino acid number of the first triplehelical amino acid is indicated to the left of the sequence. Exon boundaries and numbers are shown below the amino acid sequence of each chain. The $\alpha 1(VI)$ and $\alpha 2(VI)$ cysteine residues involved in disulfide bonding of the dimer and the $\alpha 3(VI)$ cysteine that forms disulfide bonds in the tetramers are indicated by arrows. The 19 different dominant glycine mutations that have been identified are shown above the sequence (details can be found in Supplementary Table S3). Mutations causing Bethlem myopathy (BM) or mild UCMD are black, and those identified in patients with moderate or severe UCMD are indicated in red. The two Bethlem myopathy mutations that do not measurably interfere with disulfide bonding of the tetramers are shown inside a triangle. Circled mutations interfere with intracellular disulfide bonding, but only disulfide-bonded tetramers are secreted. Both assembly phenotypes lead to deposition of normal amounts of collagen VI in the extracellular matrix and a mild phenotype. Boxed mutations lead to secretion of nondisulfide-bonded tetramers and a severe reduction in the collagen VI extracellular matrix, producing severe disease.