Kuskokwim Syndrome, a Recessive Congenital Contracture Disorder, Extends the Phenotype of FKBP10 Mutations

Aileen M. Barnes, Geraldine Duncan, MaryAnn Weis, William Paton, Wayne A. Cabral, Edward L. Mertz, Elena Makareeva, Michael J. Gambello, Felicitas L. Lacbawan, Sergey Leikin, Andrzej Fertala, David R. Eyre, Sherril J. Bale, and Joan C. Marini

ABSTRACT: Recessive mutations in FKBP10 at 17q21.2, encoding FKBP65, cause both osteogenesis imperfecta (OI) and Bruck syndrome (OI plus congenital contractures). Contractures are a variable manifestation of null/missense FKBP10 mutations. Kuskokwim syndrome (KS) is an autosomal recessive congenital contracture disorder found among Yup’ik Eskimos. Linkage mapping of KS to chromosome 17q21, together with contractures as a feature of FKBP10 mutations, made FKBP10 a candidate gene. We identified a homozygous three-nucleotide deletion in FKBP10 (c.877_879delTAC) in multiple Kuskokwim pedigrees; 3% of regional controls are carriers. The mutation deletes the highly conserved p.Tyr293 residue in FKBP65’s third peptidyl-prolyl cis-trans isomerase domain. FKBP10 transcripts are normal, but mutant FKBP65 is destabilized to a residual 5%. Collagen synthesized by KS fibroblasts has substantially decreased hydroxylation of the telopeptide lysine crucial for collagen cross-linking, with 2%–10% hydroxylation in probands versus 60% in controls. Matrix deposited by KS fibroblasts has marked reduction in maturely cross-linked collagen. KS collagen is disorganized in matrix, and fibrils formed in vitro had subtle loosening of monomer packing. Our results imply that FKBP10 mutations affect collagen indirectly, by ablating FKBP65 support for collagen telopeptide hydroxylation by lysyl hydroxylase 2, thus decreasing collagen cross-links in tendon and bone matrix. FKBP10 mutations may also underlie other arthrogryposis syndromes.

KEY WORDS: osteogenesis imperfecta; contractures; FKBP65; FKBP10; Bruck syndrome; cross-linking

Introduction

Kuskokwim syndrome (KS; MIM #208200) is an autosomal recessive congenital contracture disorder that occurs solely among Yup’ik Eskimos in and around the Kuskokwim River delta region of southwest Alaska. Affected individuals usually have congenital contractures of large joints, especially knees and/or elbows, and spinal, pelvic, and foot deformities; they sometimes have proximally or distally displaced patellae, due to tendon attenuation [Petajan et al., 1969]. Minor skeletal features have been reported, including mild vertebral wedging, mild to moderate scoliosis, pedicle elongation, and clubbing of the clavicle [Petajan et al., 1969; Wright, 1970]. Individuals with KS have white sclerae, normal teeth, and hearing.

The first individual identified with KS was born in 1922. The syndrome was recognized as a new disorder in 1969, and 17 patients from seven families living near Bethel and Togiak were described [Wright, 1970]. The sudden emergence of this syndrome can be explained by the history of the region and of the Yup’ik Eskimo people [Fienup-Riordan, 1991]. From the 1840s to 1880s, Bethel, a small (population about 40) centrally located village, was important for periodic tribal social gatherings among villages along the river, and also for interaction with Russian traders. At the native celebrations of religious festivals, social interactions led to genetic admixture in offspring conceived in Bethel but born in their own villages. In 1884, Moravian missionaries arrived in Bethel. As Christianity took hold under the Moravians, these gatherings were discontinued, requiring...
the Yup’ik to find mates within their own small villages, thus greatly increasing the frequency of consanguineous relationships. Mating selection was further reduced by the 1918–1919 “Spanish flu” epidemic, which cost the lives of fully half of the Yup’ik population. Some epidemic survivors who had resided in Bethel then migrated to Togiak and other distant villages. Two years later, the first child known to have KS was born. Thus, KS emerged due to the effective small population size, the shift in marriage patterns before and after the arrival of Moravian missionaries, and the population bottleneck caused by the influenza epidemic.

Under the hypothesis that the mutation causing KS arose on a single founder chromosome, we used both homozygosity mapping and linkage analysis to localize the gene to chromosome 17q12–21. Two candidate genes, COL1A1 (MIM #120150, NM_000088.3) and NOG (MIM #602991, NM_005450.4), were excluded in the 1990s. More recently, FKBP10 (MIM #607063, NM_021939.3) was identified as a candidate gene for KS based on gene location at 17q21.2 and the variable phenotype of previously identified FKBP10 mutations (MIM #610968) [Kelley et al., 2011; Setijowati et al., 2012; Shaheen et al., 2011; Shaheen et al., 2010].

FKBP10 encodes FKBP65, an endoplasmic reticulum-localized peptidyl-prolyl cis–trans isomerase (PPIase) and chaperone molecule [Ishikawa et al., 2008]. FKBP65 is the largest member of the immunophilin subfamily that binds FK506 and has four PPIase domains. FKBP65 has multiple ligands, including type I collagen and elastin [Davis et al., 1998; Zeng et al., 1998]. Mutations in FKBP10 were first identified as causing autosomal recessive type XI osteogenesis imperfecta (OI) in 2010 [Alanay et al., 2010]. Affected individuals had severe progressive deformity or moderately severe OI. The FKBP10 phenotype rapidly expanded to include Bruck syndrome 1, which is severe OI with congenital contractures [Kelley et al., 2011; Schwarze et al., 2013; Setijowati et al., 2012; Shaheen et al., 2011; Shaheen et al., 2010].

We have identified homozygosity for a small deletion in exon 5 of FKBP10 as the cause of KS, extending the phenotypic spectrum of FKBP10 mutations from OI alone, and OI with contractures (Bruck syndrome), to a predominantly congenital contracture syndrome. We demonstrate that the collagen-related mechanism of KS involves decreased hydroxylation of the collagen telopeptide lysine crucial for cross-linking of collagen monomers into fibrils, resulting in decreased deposition of mature collagen in matrix, and a looser appearance of fibrils formed in vitro.

Materials and Methods

Patient and Control Samples

Eight families with KS, including 11 affected individuals from whom DNA was attire, were initially ascertained by a search of the Indian Health Service database in Anchorage, AK (Supp. Fig. S1). An additional four families were ascertained by W.P., to a total of 16 affected individuals, six probably/mildly affected individuals, and 47 unaffected first-degree relatives of patients. Patients were examined either in the villages or Anchorage by S.J.B., G.D., and W.P. (under a protocol approved by the Indian Health Service, the Yukon-Kuskokwim Health Corporation, and the Yup’ik Tribal Council), or at the National Institutes of Health (NIH), under institutional review board-approved protocol 97-HD-0089. Buccal swabs were also collected from individuals in Alaska, and EDTA blood from patients seen at NIH. Buccal swabs were collected from 48 unaffected, unrelated Yup’ik individuals to serve as ethnically matched controls.

Clinical Examinations

Patients seen in Alaska had detailed reviews of medical records, brief physical examinations, and buccal swab collection. Three families with five probands (Fig. 1A) underwent more extensive examinations. The five patients seen at the NIH underwent physical examinations including dermatology, physical medicine and rehabilitation, physical and occupational therapy, as well as electrocardiogram, audiometry, and skin biopsy. Laboratory studies included
complete blood count and serum chemistry, radiographic skeletal survey, MRI of knees and elbows, and bone mineral density (DXA). Ultrasound examination of patellar and elbow tendons was performed on several patients.

**Linkage Mapping**

DNA was extracted from buccal swabs and blood, as per standard protocols. Three DNA pools were prepared as follows: pool 1: 11 affected individuals; pool 2: 17 obligate heterozygotes (parents and offspring of affected individuals); pool 3: 20 unaffected, unrelated Yup’ik controls. The pooled samples were screened using 387 polymorphic markers, with average heterozygosity of 76% and average spacing of 10 cM (version 8, CHLC Human Screening Set, Research Genetics, Huntsville, AL), and examined by incorporating 32P in amplification reactions. The markers that showed a reduction in the number of alleles in the affected pool, as compared with the control pool, were verified by typing of individual family members and by analysis of other nearby markers.

**PCR and Sequencing**

Genomic DNA (gDNA) was prepared from fibroblasts or leukocytes using a Gentra Puregene DNA extraction kit (Qiagen, Valencia, CA). Proband fibroblast gDNA was used to amplify the 10 exons of FKBP10 (NM_021939.3) by PCR. PCR products were then sequenced (Macrogen Corp., Rockville, MD) and compared with the expression levels of other nearby markers.

**Real-Time PCR**

Total RNA was extracted from primary fibroblasts using TriReagent (Molecular Research Center, Cincinnati, OH); RNA integrity was verified on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Complementary DNA was reverse transcribed from 5 μg RNA using a High Capacity cDNA Archive Kit (Applied Biosystems/Life Technologies Corporation, Carlsbad, CA). The relative expression of FKBP10 was assessed by comparing it with the expression of GAPDH, using Taqman Gene Expression Assays (Applied Biosystems/Life Technologies Corporation, Carlsbad, CA). Expression levels were normalized to control primary fibroblasts.

**Western Blot**

Cell lysates from proband fibroblasts were collected in radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Proteins were separated on precast 4%–15% ready gels (Bio-Rad, Hercules, CA), transferred to 0.2 μm nitrocellulose membranes and blocked with 5% bovine serum albumin plus 1× casein before probing with FKBP65 (Abnova, Taipei, Taiwan) and actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies overnight. Blots were washed, incubated with secondary infrared-conjugated antibodies for 1 hr, washed, and visualized and quantified on a LI-COR Odyssey infrared imager (LI-COR, Lincoln, NE).

**Steady-State Collagen**

For type I collagen studies, control and proband fibroblasts were grown to confluence in six-well culture dishes. Steady-state collagen analysis was conducted as previously described [Cabral et al., 2005]. Cells were incubated with 437.5 μCi/ml L-[2,3,4,5-3H] proline for 16–18 hr, prior to collection and ammonium sulfate precipitation, then peptin digested and analyzed by 6% SDS-urea-PAGE.

**Differential Scanning Calorimetry**

Differential scanning calorimetry (DSC) of secreted collagen solutions in 0.2 M sodium phosphate and 0.3 M glycerol at pH 7.4 from proband K5-II.8 and null cells was performed from 10°C to 55°C in a Nano III DSC instrument (Calorimetry Sciences Corporation, Lindon, UT), as previously described [Makareeva et al., 2008].

**Collagen Secretion**

Collagen secretion rate was measured in the presence of ascorbic phosphate and 0.5% serum as described before [Makareeva et al., 2010]. Absolute collagen concentration was quantified by adding AlexaFluor488-labeled MMP-1 fragments of type I mouse tail tendon collagen as an internal standard followed by Cy3-labeling of the mixture and separated on 3%–8% gradient Tris-Acetate mini gels (Invitrogen/Life Technologies, Grand Island, NY). The collagen to internal standard ratio was measured by densitometry of gel fluorescence on an FLA5000 scanner (Fuji Medical Systems, Tokyo, Japan), which was analyzed with Multi-Gauge software supplied with the scanner (Fuji Medical Systems, Tokyo, Japan). The secretion rate was normalized to the number of cells in the well.

**Matrix Deposition**

For extracellular matrix deposition assays, postconfluent cells were stimulated with 100 μg/ml ascorbic acid three times a week for 14 days. After 14 days, fibroblasts were either labeled for 24 hr with 406.25 μCi/ml L-[2,3,4,5-3H] proline and the media and cell layer were harvested, as described previously [Bateman and Golub, 1994], or used for Raman microspectroscopy. The 3H-proline-labeled collagens were sequentially extracted from the cell-layer fraction with neutral salt, acetic acid, and peptin. These matrix extracts were run on 6% SDS-urea-PAGE. Collagen content of each fraction was measured by densitometry and normalized to sample volume.

**Immunofluorescence Microscopy**

For FKBP65 and type I collagen immunofluorescence staining, fibroblasts were plated on two-well chamber slides (Nalge Nunc International, Rochester, NY), grown for 48 hr and stained. Briefly, for costaining of FKBP65 (BD, Franklin Lakes, NJ) and LF-68 (a1[1] C-telopeptide; a generous gift from Dr. Larry Fisher, NIH) cells were washed with PBS, then fixed in 4% parafomaldehyde. Fixed cells were washed, permeabilized on ice in 1% goat serum plus 0.2% TritonX-100 for 5 min, washed again and blocked with 1% BSA in PBS. Cells were incubated with primary antibody for 1 hr, washed in PBS, and incubated with Alexa Fluor-555 and Alexa Fluor-488-conjugated secondary antibodies for 1 hr then washed extensively. Slides were mounted using mounting media with 4’,6-diamidino-2-phenyl indole (DAPI) (Vector Laboratories, Burlingame, CA) and coverslips. Stained cells were imaged using a Zeiss LSM 510 Inverted Meta microscope and LSM510 software (Carl Zeiss, Oberkochen, Germany).

Staining of the extracellular matrix was performed essentially as described [Valli et al., 2012]. Cells were grown to confluence on chamber slides, then treated with DMEM+10% fetal bovine serum.
containing 100 μg/ml ascorbic acid three times a week for 2 weeks. Matrices were washed in PBS, then fixed in 4% paraformaldehyde. The matrix was blocked in 1% BSA in PBS plus 0.02% Tween-20, washed with PBS and incubated with primary antibody (LF-68) in 1% BSA/PBS for 1 hr. After washing, the matrix was incubated with secondary antibody for 1–1.5 hr, washed, and examined as above.

**Raman Microspectroscopy**

Confluent cell cultures fixed in 1% formaldehyde in PBS for 4 hr at 37°C were hydrated with PBS supplemented with 1 ng/ml DAPI stain for nuclei and examined in a Senterra confocal Raman microscope (Bruker Optics, Billerica, MA) as described before [Barnes et al., 2012]. (Auto)Fluorescence images of the pieces were taken under 365 nm light to locate cell cytoplasm. The excitation laser was focused to 2 × 2 μm² areas pointed at the cytoplasm (between nuclear edge and cell periphery). Raman-scattered light was collected from a 7 μm high focal volume, which encapsulated both cells and their matrix. Raman scattering spectra with 10–15 cm⁻¹ resolution were collected from different 140 × 170 μm² regions within each culture, averaged over 10 points within each region, and corrected for water and quartz contributions as well as for 1–6 cm⁻¹ instrumental variability in frequency calibration. Matrix collagen to cell organics ratios were evaluated from decomposition of the corrected spectra on collagen-free cell cytoplasm and of purified collagen, normalized to have similar integral intensities of stretching vibrations of all organic CH groups. Decomposition was in the regions of the CH stretching (2,780–3,040 cm⁻¹) or in the amide III region (1,180–1,380 cm⁻¹) where collagen and cells have very distinct spectra. Alternatively, the spectra were decomposed from ratios of integral intensities of baseline-corrected, spectral bands stretching of all organic CH groups. Decomposition was in the regions of the CH stretching (2,780–3,040 cm⁻¹) or in the amide III region (1,180–1,380 cm⁻¹) where collagen and cells have very distinct spectra. Alternatively, the spectra were decomposed from ratios of integral intensities of baseline-corrected, spectral bands dominated by (hydroxy)proline (832–893 cm⁻¹) to those of CH stretching (2,810–3,028 cm⁻¹) vibrations dominated by proteins and lipids. Content of intracellular collagen in these cultures was negligible. Standard errors and P values (two-tail t-test) were calculated for variations between different regions within the control (n = 14) and mutant (n = 6–8) cultures.

**Amino Acid Analysis**

Amino acid analysis to quantify 4-hydroxyproline, proline, hydroxylysine, and lysine was performed by high-pressure liquid chromatography (ALLBiotech, Richmond, VA). Telopeptide lysyl hydroxylation of COL1A1 p.Lys1208 was assessed by ion-trap mass spectrometry. Secreted procollagen was precipitated by 1 M (NH₄)₂SO₄, from which pro-collagen chains were resolved by SDS-PAGE and subjected to in-gel trypsin digestion for analysis of targeted peptides by electrospray mass spectrometry.

**In Vitro Fibrillogenesis**

To analyze the morphology of collagen fibrils formed from collagen molecules with aberrant hydroxylation of telopeptides, we employed de novo fibril formation assays with the use of procollagen I isolated from the cultures of probands’ dermal fibroblasts, as described earlier [Cabral et al., 2007; Steplewski et al., 2004]. In brief, fibril formation was initiated by processing procollagen I at 150 μg/ml with endopeptidase Lys-C (EC.3.4.21.50, endoproteinase Lys-C, Roche, Basel, Switzerland) at 37°C. Note: As Lys-C cleaves proteins at the C-terminal side of the lysine residues, employing this enzyme to process procollagen I propeptides conserves a critical lysine residue of the C-terminal telopeptide of the α1(1) chain (p.Lys1208) but not the lysine residues present within the N-terminal telopeptides.

To preserve the structure of newly formed fibrils and to minimize their aggregation, the fibril formation process was carried out in a hanging drop according to a described method [Fertala et al., 1996]. In brief, samples of 5 μl of mixture containing procollagen I and Lys-C were transferred to a Teflon-insulated wire to form a hanging drop; the wire was mounted inside a screw cap, and then it was placed above a buffer in a tightly closed tube. The tube was incubated at 37°C for 24 hr and then the whole drop was placed on a carbon-coated copper grid (Ted Pella Inc., Redding, CA) and left to adsorb for 2 min. Subsequently, collagen fibrils were negatively stained with 1% phosphotungstic acid (Ted Pella Inc., Redding, CA). The negatively stained samples were analyzed using a Hitachi H-7000 transmission electron microscope (Hitachi, Tokyo, Japan).

**RESULTS**

**Clinical Phenotype**

KS patients usually have congenital contractures, especially of lower extremities, which progress during childhood and persist for the lifetime of the individual. Not all patients with KS have contractures at birth, reflecting the variability of contractures with FKBP10 mutations. The severity of contractures can be strikingly asymmetrical in any given homozygous individual. The knees and elbows are frequently involved. Bony deformities of the spine, pelvis, and feet are frequently seen. Occasional patients with full bilateral contractures of the knees ambulate either by “duck walking” (hunkering), where the individuals sit with buttocks on their heels, or by “knee walking”, where they amble on their knees with their lower legs drawn up behind them to their buttocks [Petajan et al., 1969], although most are treated with leg braces and/or surgery in childhood and walk upright. Hip flexion contractures can be masked by lumbar hyperlordosis. Foot and ankle involvement included internal tibial torsion and planovalgus or equinovarus deformities. There may be associated muscle atrophy of limbs with contractures, and proximal or distal displacement of patellae due to attenuation of patellar or quadriceps tendons. Proximal patella displacement, when it occurs, may be progressive in childhood, but is present only in some severe individuals. Distal displacement is a subtle, but more common, finding and is a good indicator of clinically significant contractures. Mild skeletal features are common, with osteopenic or osteoporotic vertebrae (L1–L4 z-score from –0.48 to –2.29) and mild vertebral wedging. X-rays reveal severe lumbar lordosis with associated horizontal sacrum, severe sacral kyphosis (hooked sacrum), superior angulation of the transverse processes of the lower lumbar vertebrae, and elongation of the lumbar pedicles. Spondylolisthesis and mild to moderate scoliosis are occasionally seen (Fig. 1B–C, Supp. Figs. S2 and S3). Lordosis is usually much less apparent on physical exam than on X-ray, being masked by pedicle elongation. Affected individuals have marked lordosis with the sacrum position being nearly horizontal (Supp. Fig. S3). They tend to have barrel chests. Many probands have incurred several low energy fractures, including femora. In more severe cases, protrusio acetabulæ occurs. Affected individuals may also have hallux valgus, planus valgus feet, or talipes equinovarus. A very consistent X-ray finding is clubbing of the distal clavicle.

Individuals with KS have white sclerae and normal dentition and hearing. Probands have relatively short stature, with height below the fifth percentile for age (50th percentile for 9.5–15 years old) when compared with the standard US growth tables, but are less

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short compared with other Yup’ik people. Head circumference is macrocephalic.

The detailed descriptions of the probands in the Supporting Information show the variability of symptoms among affected individuals, even within sibships. Among probands examined in detail, probands 4 and 5 (K3-III.6 and K5-II.8) have more severe symptoms and findings, whereas proband 1 (K2-II.1) is intermediate and probands 2 and 3 (K2-II.2 and K3-III.5) are relatively mild. Probands 1 and 2 are siblings, as are probands 3 and 4, showing variability even with sibships.

Mapping of KS to 17q12–21

Twelve families, residing in eight Yup’ik villages and two towns, were ascertained. Homozygosity mapping using pooled DNA from affected individuals showed only a single allele for three markers on chromosome 17q21 (D17S800, KRT9-CA repeat, and D17S846), whereas control and relative pools showed multiple alleles (Supp. Table S1). On radiation hybrid mapping, these three markers gave logarithm of odds (LOD) scores ranging from 8.58 to 12.8, confirming the likelihood of the mutation in this region. Patients and family members were all typed individually for the three conserved markers, revealing homozygosity in all affected for a “4–4–1” haplotype. Of six “probably/mildly” affected individuals included in the analysis, five were homozygous 4–4–1, and one carried one 4–4–1 haplotype and one 4–4–2 haplotype. Flanking markers on the centromeric side showed the presence of multiple alleles in the affected pool, originally identifying a conserved haplotype of approximately 0.83 Mb on chromosome 17q21. When the conserved region was first defined, the D17S1793 marker qter to this region was not examined in all individuals. In retrospect, the available data on D17S1793 indicate that this marker should be included in the conserved haplotype.

In 1999, COL1A1 (17q21.33) was explored as a candidate gene; however, radiation hybrid mapping placed COL1A1 distal to the conserved haplotype and the COL1A1 sequence was normal in affected individuals.

FKBP10 Mutation in Kuskokwim Pedigrees

Because the updated KS homozygous region included FKBP10 at 17q21.2, in which congenital contractures had been shown to be a variable feature of recessive type XI OI [Shaheen et al., 2010], we sequenced the 10 exons of FKBP10 in K3-III.6 fibroblast DNA. We identified a homozygous three-nucleotide deletion in FKBP10 exon 5 (c.877_879delTAC, Fig. 2A), which causes deletion of a single residue, p.Tyr293. This deletion occurs in the 3rd PPlase domain of FKBP65, at a highly conserved residue (Fig. 2B). The mutation eliminates an Msl restriction site, which was used to confirm the presence of homozygous deletion in probands with varying clinical severity from three Kuskokwim pedigrees (Fig. 2C). Furthermore, DNA samples from 48 unaffected controls from the Kuskokwim delta region were examined; about 3% (3/96 alleles) were

Figure 2. Kuskokwim mutation in FKBP10. A: Sequencing tracing of FKBP10 c.877_879delTAC in KS proband K3-III.6 compared with normal sequence. B: Conservation of p.Tyr293 across various species. The three-nucleotide deletion (underline) results in the deletion of p.Tyr293 (highlighted). C: Confirmation of homozygous FKBP10 c.877_879delTAC in all probands by elimination of the Msl restriction site. D: Schematic diagram of the phenotype of FKBP10 mutations. Recessive null or missense FKBP10 mutations lead to OI with or without contractures, whereas the KS in-frame FKBP10 deletion leads to a contracture phenotype. E: Western blot of proband RIPA cell lysates showing residual FKBP65 protein in all probands (for quantitation, see Table 1). β-Actin is used as a loading control.
Table 1. Effect of the FKBP10 Mutation on Collagen

<table>
<thead>
<tr>
<th>mRNA expression</th>
<th>K2-II.1</th>
<th>K2-II.2</th>
<th>K3-III.5</th>
<th>K3-III.6</th>
<th>K5-II.8</th>
<th>Null</th>
<th>Control</th>
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<tr>
<td>FKBP10</td>
<td>1.14 ± 0.12</td>
<td>1.67 ± 0.18</td>
<td>0.79 ± 0.09</td>
<td>1.07 ± 0.20</td>
<td>1.59 ± 0.42</td>
<td>0.06 ± 0.01</td>
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<tr>
<td>FKBP65</td>
<td>5.58 ± 2.38</td>
<td>3.46 ± 1.14</td>
<td>2.68 ± 0.37</td>
<td>3.13 ± 1.46</td>
<td>6.49 ± 2.88</td>
<td>&lt;0.01</td>
<td>100</td>
</tr>
<tr>
<td>Hydroxylation (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyd/total Lys (% difference from control)</td>
<td>35</td>
<td>37</td>
<td>22</td>
<td>12.75</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyd/total Pro (% difference from control)</td>
<td>-5</td>
<td>-7</td>
<td>-1</td>
<td>+7</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Telopeptide lysyl hydroxylation (%)</td>
<td>5</td>
<td>8</td>
<td>10</td>
<td>3</td>
<td>2</td>
<td>&lt;1</td>
<td>58-59</td>
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<td>Collagen secretion</td>
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<tr>
<td>pg collagen/hr cell</td>
<td>1.35 ± 0.33</td>
<td>1.51 ± 0.32</td>
<td>1.48 ± 0.09</td>
<td>1.54 ± 0.11</td>
<td>1.59 ± 0.24</td>
<td>0.87</td>
<td>1.13 ± 0.36</td>
</tr>
</tbody>
</table>

Values are means ± SD, n = 2–3. The relative levels of mRNA expression were normalized to GAPDH expression, and then compared with the level of expression in control fibroblasts (arbitrarily set to 1). The relative levels of protein were normalized to actin, and then compared with normal protein levels from a control cell line (arbitrarily set to 100).

FKBP10 Expression and FKBP65 Stability in KS

Transcript levels of FKBP10 in fibroblasts from five Kuskokwim probands who were homozygous for the in-frame deletion were in the normal range (79%–167% of control) (Table 1). Expression levels varied as much between siblings as between pedigrees, but did not correlate with severity.

The in-frame deletion destabilizes the FKBP65 protein. Only residual FKBP65 was detected on Western blots of proband fibroblast lysates (2.68%–6.44% of control, Fig. 2E).

Effect of KS FKBP10 Mutation on Collagen Synthesis, Secretion, and Stability

We examined type I collagen biochemistry to determine the effect of decreased FKBP65 on collagen posttranslational modification and metabolism. Steady-state collagen synthesized by probands K3-III.5 and K3-III.6 fibroblasts showed normal electrophoretic migration with a slight band broadening (Fig. 3A), consistent with a minimal increase in collagen melting temperature on DSC (Fig. 3B). Amino acid analysis of the collagen helix by liquid chromatography revealed a 30% increase in the number of hydroxylated lysines (Table 1). On average, this is equivalent to hydroxylation of an additional two or three lysines in a collagen monomer versus control samples.

The absolute amount of collagen secreted into media by proband fibroblasts in culture was determined with an assay using internal collagen standards. Collagen secretion (pg/hr/cell) was normal by t-test, ranging from 119% to 141% of control cell values (Table 1). Intracellular collagen showed no evidence of aggregation on immunofluorescence microscopy (Fig. 3C).

Effect of KS FKBP10 Mutation on Collagen Deposition

Collagen deposition into matrix was examined biochemically, as well as by confocal microscopy and Raman microspectroscopy. In the biochemical assay, fractions were successively extracted from the deposited matrix by neutral salt, which contains newly incorporated collagen without cross-links; acetic acid, which contains collagen with immature cross-links; and pepsin, which contains collagen with mature cross-links. Fibroblasts from individuals with relatively milder (K3-III.5) and more severe (K3-II.8) KS were utilized. There was a consistent decrease of collagen deposited into the maturely cross-linked fraction, ranging from half to one-sixth of control. In addition, the β-forms in the cross-linked fractions were clearly reduced, with β1.1 and β2.2 nearly absent and only β1.2 well detected (Fig. 4A).

On confocal microscopy, the collagen deposited by fibroblasts homozygous for the Kuskokwim FKBP10 mutation was generally more sparse and disorganized than control and contained spindly structures with bright edges (Fig. 4B). The amount of collagen in matrix was intermediate between the control and FKBP10-null cells. Further examination of deposited matrix by Raman microspectroscopy revealed variable collagen deposition in KS matrix, ranging from one-third to normal levels, with no correlation with disease severity (Fig. 4C). Raman microspectroscopy confirmed the decreased deposition in K3-III.5 and K5-II.8 in culture, but two other KS probands had normal matrix deposition by Raman.

Collagen Telopeptide Lysine Crucial for Cross-Linking has Reduced Hydroxylation

The reduced deposition of mature collagen into matrix in culture led us to examine the hydroxylation status of the type I collagen C-telopeptide lysine, which is crucial for cross-linking of collagen into matrix, using mass spectrometry of tryptic peptides (Table 1). Although telopeptide residues are not normally hydroxylated in skin tissue, this residue is 60% hydroxylated in cultured normal fibroblasts and has been shown to be <1% hydroxylated in FKBP10-null cells [Barnes et al., 2012]. In KS samples, hydroxylation of COL1A1 p.Lys1208 was found to be significantly reduced, although not absent, ranging from 2% to 10%. More mildly affected individuals had slightly greater levels of telopeptide hydroxylation than more severely affected individuals, 8%–10% versus 2%–3%.

In Vitro Fibrillogenesis of KS Collagen Yields Looser Collagen Fibrils

In vitro fibrillogenesis of probands K3-III.5 and K5-II.8 secreted collagen yielded fibrils with a normal D-periodicity. The presence of collagen molecules with decreased content of hydroxylated p.Lys1208 was associated with less tightly bundled fibrils, with evidence of splitting and splaying of the fibers (Fig. 5A). Transmission electron microscopy of collagen fibrils from K3-III.6 skin had cross sections that were less rounded than control, whereas longitudinal fibrils had a normal D-periodicity (Fig. 5B).
Discussion

In this report, we have identified the causative mutation of KS, a congenital contracture disorder occurring among Yup’ik Eskimos, as a three-nucleotide deletion in \( \text{FKBP10} \), resulting in loss of the highly conserved p.Tyr293 residue of FKBP65. Linkage mapping localized KS to chromosome 17q12–21 in the 1990s and several potential candidate genes, including \( \text{COL1A1} \), were ruled out at that time. Subsequently, null and missense mutations in \( \text{FKBP10} \) were shown to be the cause of moderate to severe recessive OI, as well as Bruck syndrome 1, which is severe OI with congenital contractures [Alanay et al., 2010; Cabral et al., 2005; Kelley et al., 2011; Puig-Hervas et al., 2012; Setjiowati et al., 2012; Shaheen et al., 2011; Shaheen et al., 2010; Steinlein et al., 2011; Venturi et al., 2012].
Because the same FKB10 mutation may cause OI alone or OI with contractures in unrelated individuals or even in siblings, contractures are now understood to be a variable manifestation of FKB10 mutations. The combination of variable contractures in the FKB10 phenotype and the location of FKB10 at 17q21.2 made FKB10 a prime KS candidate gene. All tested probands with KS are homozygous for the p.Tyr293 deletion, and ~3% of ethnically matched normal controls are heterozygous carriers. This discovery will provide carrier and prenatal testing to the Kuskokwim community, via a simple and inexpensive blood test. Should the Yup’ik community so choose, they now have the potential to allow the elimination of this syndrome in a generation or two. In addition, this mutation extends the phenotypes associated with recessive FKB10 mutations in a manner that complements the existing phenotypes, so that an individual homozygous for a null or missense mutation will have either OI or Bruck syndrome, whereas an individual homozygous for deletion of the p.Tyr293 residue will have a congenital contracture condition with milder skeletal findings (Fig. 2D).
In vitro and dermal collagen fibrils from KS probands. β-FKBP10 deficiency is not a function performed directly by FKBP65. FKBP10 mutations and their highly prevalent occurrence in KS are mutationally regulated, leading to essentially no hydroxylation of the type I collagen telopeptide lysine. Affected individuals with KS have variable and severe skeletal manifestations, including normal to osteopenic bone density, scoliosis and lordosis, short stature, and low energy fractures. However, these findings are likely due in part to abnormal and restricted mobility from their contractures and did not attain a severity that previously merited an OI designation. Apparently, residual FKBP65 and COL1A1 Lys1208 hydroxylation do not significantly alter the ability of collagen molecules to self-assemble into fibrils and is sufficient to surpass a threshold crucial for osteoblasts to deposit and cross-link collagen well enough to support bone structure. In fact, KS fibroblasts deposit an intermediate level of collagen into matrix in vitro, between normal and FKBP10-null cells. Furthermore, this correlation is supported by the clinical findings of the KS probands examined extensively at the NIH, in which probands K3-II.6 and K3-II.8, who have 2%–3% residual telopeptide hydroxylation, have more significant skeletal symptoms than that of probands K2-II.2 and K3-II.5, with 8%–10% hydroxylation, notwithstanding the sibling relationship between the K3 probands.

Interestingly, the collagen-related function that is ablated by FKBP10 deficiency is not a function performed directly by FKBP65. Lysyl hydroxylase 2 (LH2), encoded by PLOD2 (NM_182943.2), hydroxylates the collagen telopeptide lysine. It is possible that the speculation applied to tropoelastin [Davis et al., 1998] concerning steric hindrance applies to the type I collagen telopeptide, that is, prolines that have not been isomerized and are located close to a lysine will block the access of LH2. In the primary sequence of collagen, the nearest prolines are amino to the telopeptide lysine and are located in the triple helical region. Steric hindrance of LH2 would then need to involve transient-altered folding of the helical region, which is not supported by evidence. The more likely alternative is that FKBP65 is crucial in some way for the stability or activity of LH2, as previously proposed [Barnes et al., 2012; Schwarze et al., 2013]. This might involve complex formation, or simply LH2 as a substrate ligand of FKBP65.

Decreased collagen cross-links in bone likely underlie the bone fragility component of the FKBP10 phenotype [Barnes et al., 2012; Schwarze et al., 2013]. Cross-links of collagen in bone are decreased in postmenopausal osteoporosis [Bailey et al., 1993; Oxlund et al., 1996]. Treatment of animal models with β-aminopropionitrile to totally inhibit cross-link formation results in weakened bone [Oxlund et al., 1995; Oxlund et al., 1996]. Additionally, the phenotype is related to that found in Bruck syndrome 2, a recessive condition caused by absence of the enzyme LH2 [Ha-Vinh et al., 2004].

The variable occurrence of contractures with null and missense FKBP10 mutations and their highly prevalent occurrence in KS are more difficult to explain. Even in KS, some homozygous individuals lack contractures into adulthood. The descriptive clinical term for the occurrence of multiple congenital joint contractures that limit ambulation is arthrogryposis; KS was originally called “arthrogryposis of the Eskimo.” Several dozen arthrogryposis entities have been classified with a total population prevalence estimated between one in 3,300 to one in 5,100. The contractures are found in the upper and lower extremities and are often symmetric. A common mechanism for the contractures is absence or decrease in the movement normally required in utero for correct formation of tissue around the joints. Large joints develop in a flexion position, so if in utero movement is decreased by neurological (central or noncentral), muscular, or vascular conditions, then extra tissue will form around the joint (the Swinyard phenomenon) and fix the flexion position. Presuming that the immediate cause of contractures in FKBP10 mutations is decreased in utero movement, the originating cause could be an abnormality in the strength or organization of the connective tissue. In mice, FKBP10 was shown to be developmentally regulated, as 12-day-old mice had higher expression in aorta, brain, kidney, and lung when compared with near absent expression in adult mice [Patterson et al., 2000]. Additionally, FKBP10 is upregulated at sites of active matrix production [Patterson et al., 2005] and therefore its mutation may affect the quality of extracellular matrix that is produced, both during development and throughout life.

Since congenital contractures are not seen in other types of OI, we are led to hypothesize that lack of collagen telopeptide
hydroxylation must have a special consequence in tendon collagen. In the absence of tendon tissue for examination, we reflect on the distinctive pathway for collagen secretion into tendon matrix, involving linear secretion and deposition through fibrisepotors [Canty et al., 2004]. Disturbance of this very directional process by lack of telopeptide hydroxylation could account for this special feature of FGKBP10 mutations. It is also possible that the level of modifying enzymes such as LH2 might be different in tendon than in dermis or bone, adding to the susceptibility of this tissue. Finally, one wonders whether the phenotypes of FGKBP10 mutations might not be further extended by targeted examination of this gene in other congenital contracture conditions.

Acknowledgments

We would like to acknowledge the National Institute of Child Health and Human Development Microscopy and Imaging Core, in which the confocal microscopy was conducted.

Disclosure statement: The authors declare no conflicts of interest.

References


