Mutations that alter the carboxy-terminal-propeptide cleavage site of the chains of type I procollagen are associated with a unique osteogenesis imperfecta phenotype

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Abstract

Osteogenesis imperfecta (OI) is a genetic bone disorder characterised by fractures, low bone mass, and skeletal fragility. It most commonly arises from dominantly-inherited mutations in the genes *COL1A1* and *COL1A2* that encode the chains of type I collagen. A number of recent reports have suggested that mutations affecting the carboxyl-terminal propeptide cleavage site in the products of either *COL1A1* or *COL1A2* give rise to a form of OI characterized by unusually dense bones. We have assembled clinical, biochemical, and molecular data from 29 individuals from 8 families with 7 different mutations affecting the C-propeptide cleavage site.

The phenotype was generally mild: the median height was ~33th centile. Eighty percent of subjects had their first fracture by the age of 10; and a third had a femoral or tibial fracture by the age of 25. Fractures continued into adulthood, though rates varied considerably. Healing was normal, and rarely resulted in long bone deformity. A third of subjects over 15 years old had scoliosis. The teeth and hearing were normal in most and blue sclerae were not observed. Other features noted included fibro-osseous dysplasia of the mandible and Achilles tendon calcification. The mean spinal bone mineral density z-score was +2.9 (SD 2.1) compared to -2.2 (0.7) in subjects with *COL1A1* haploinsufficiency mutations. Bone mineral density distribution, assessed by quantitative backscattered electron imaging in bone showed higher levels of mineralization than seen in any other disorder. Bone histology showed high trabecular volume and increased cortical thickness, with hyperosteoidosis and delayed mineralization. *In vitro* studies with cultured skin fibroblasts suggested that these mutations interfere with processing of the chain in which the sequence alteration occurs, but the C-propeptide is eventually cleaved (and detectable in blood), suggesting there are alternative sites of cleavage. The precise mechanism of the bony pathology is not yet clear.

Introduction

Osteogenesis imperfecta (OI) is a genetic bone disorder characterised by fractures, low bone mass, and skeletal fragility. It most commonly arises from dominantly-inherited mutations in the genes (*COL1A1* and *COL1A2*) that encode the chains of type I collagen - the most abundant and main structural protein of bone.

Type I collagen is initially synthesized as a precursor molecule (type I procollagen) that comprises two proα1(I) and one proα2(I) peptide chains (encoded by *COL1A1* and *COL1A2*, respectively). Proα1(I) and proα2(I) have similar structures, with a core triple-helical domain of 1,014 amino acids composed of uninterrupted Gly-Xaa-Yaa tripeptide repeats, flanked by propeptides at both the amino-terminal (N) and carboxyl-terminal (C) ends. During and after translation the three chains undergo extensive modification necessary for thermal stability and the formation of stable, complex, intermolecular cross-links. Directed by sequences in the carboxyl-terminal domain, these chains then combine in a 2:1 ratio into a trimeric structure with propagation of the triple helical collagen structure in a C- to N-terminal direction. Following secretion into the pericellular domain the N-terminal and C-terminal propeptides are cleaved by proteases (encoded by *ADAMTS2* and *BMP1*, respectively). Cleavage of the C-propeptide occurs at an alanine-aspartate dipeptide in each chain [residues 1218-1219 in proα1(I) and 1119-1120 in proα2(I)] [1]. The cleaved propeptides, measured in plasma as procollagen-1 N-propeptide (P1NP) and procollagen-1 C-propeptide (P1CP), are used as bone formation markers.

There are two general classes of mutations in *COL1A1* and *COL1A2* that cause OI: those that result in a quantitative defect with synthesis of structurally normal type I procollagen at about half the normal amount (haploinsufficiency), and those that result in synthesis of a structurally abnormal collagen. Haploinsufficiency mutations are usually the consequence of premature termination codons in one *COL1A1* allele that initiate nonsense-mediated decay of the mRNA from the affected allele. These generally result in a mild, non-deforming phenotype with blue sclerae (OI type I in the Sillence classification). Mutations that change the protein sequence in the triple-helical domain produce a wide phenotypic range (from mild to lethal). Most prevalent are missense mutations that result in substitution for the invariant triple helical glycine residues that permit triple helix formation. Others cause

alterations in splice sites that can lead to exon skipping, intronic inclusion, or activation of cryptic sites in introns or exons that retain the reading frame [1]. Other than these two broad categories, specific genotype-phenotype relationships in OI have proven difficult to establish [2-4].

In 2007 Lindahl *et al* described a 13 year-old girl with a mild OI phenotype and unusually high bone mass on dual energy x-ray absorptiometry (DXA) scanning. She had a pathogenic variant (c.3355 G>A) that led to substitution of the aspartic acid at position 1219 of the protein by asparagine (p.Asp1219Asn) that altered the C-propeptide cleavage site of in the proα1(I) chains encoded by the altered *COL1A1* allele [5]. In 2008, we described a family with OI with a *COL1A1* variant (c.3652 G>A; p.Ala1218Thr), members of which had high bone mass that was paradoxically associated with hyperosteoidosis [6]. Further reports in a small number of families have confirmed the association of high bone mass with mutations that affect the C-propeptide cleavage site of either *COL1A1* or *COL1A2* [7-13]. We report here data from 29 subjects from 8 different families with 7 different pathogenic missense variants that alter the C-propeptide cleavage site. It is clear that this restricted class of pathogenic variants results in a distinctive OI phenotype with variable expression.

Subjects and Methods

Subjects. We studied 29 subjects (13 male, 16 female) aged 2½ – 75 (mean 29) years from eight different families. The number of affected members in each family ranged from 1 to 10. In all cases where more than one family member was affected there was a dominant pattern of inheritance (Supplementary data, Figure 1). The mutations are listed in Table 1 and illustrated in Figure 1. Some details of families A and H have been described in abstract form [6,7]; family B has been described in a recent paper [9]; mention is made of family E in the paper of Pollitt *et al* [11] and family G in an abstract [14]. Families C, D and F have not been reported previously, but a radiograph from one subject (F3) appeared in a review article [15].

Clinical data that included fracture history and other phenotypic characteristics were collected on a standardized data sheet (Supplementary data Table 1).

Bone mineral density (BMD) was measured using dual energy x-ray absorptiometry in 22 subjects and the results expressed as SD (z-score) relative to age- and sex-specific normal mean (according to the manufacturers' data). For comparison, we also measured bone density in 23 subjects aged 5 - 53 years (mean age 21; 13 female, 10 male) with typical mild OI associated with haploinsufficiency mutations in *COL1A1*.

Biochemistry. Serum concentrations of procollagen-1 C-propeptide (P1CP) were measured in five subjects with C-propeptide cleavage site mutations (A2, A3, G1, G2 and G3) and eight subjects, aged 19-52, who had OI type I due to haploinsufficiency mutations in *COL1A1*. P1CP was measured by an enzyme-linked immunosorbent assay (Cloud-Clone Corp, Houston Texas, USA; normal range 70-165 ug/l).

Bone biopsy. Transiliac bone biopsies were taken under local anesthesia and light sedation, using an 8mm trephine in subjects A2, A3, G2 and G3 after tetracycline double-labelling with a 2/14/2 day on/off/on schedule, completed 2 days before the biopsy was taken. After dehydration in ascending concentrations of ethanol, the undecalcified samples were embedded in methylmethacrylate, and 5-µm-thick sections were cut using a microtome, deplasticized and resin removed prior to staining. These sections were used for von Kossa, Goldner trichrome and toluidine blue staining and 20-um thick sections were used for tetracycline fluorescence.

Quantitative histomorphometry was undertaken on von Kossa or Goldner trichrome-stained and unstained tetracycline fluorescence sections using the OsteoMeasure histomorphometry system (Osteometrics; Atlanta, GA, USA). The static morphometry parameters reported [16] included: trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), mean cortical width (Ct.wi), cortical porosity (Ct.po), trabecular osteoid volume (OV/BV), trabecular osteoid surface (OS/TbS), osteoid seam thickness (O.Th), trabecular osteblastic osteoid (Ob.S/BS), trabecular resorptive (eroded) surface (ER/TbS), trabecular osteoclastic surface (Oc.S/BS), and osteoclast number per mineralized bone surface (Oc.S/Md.BS), and cortical width. The dynamic morphometry parameters reported [16] included mineral apposition rate and mineralization lag time.

Bone mineralization density distribution (BMDD). Quantitative backscattered electron imaging (qBEI) was performed on biopsy samples (entire bone section areas) from two adult subjects. Full details of the method have been published previously [17,18]. The digital images were used for the evaluation of the grey-level histograms, which were further transformed to weight percent calcium histograms (so-called bone mineralization density distributions, BMDD). The following parameters were derived: CaMean: the weighted mean Ca-concentration of the bone area; CaPeak: the mode calcium concentration (the peak position of the histogram), which indicates the most frequently occurring calcium concentration of the scanned bone area; CaWidth: the full width at half maximum of the distribution, describing the variation in mineralization density; CaLow: the percentage of mineralized bone with a calcium concentration < 5th centile of the reference BMDD of cancellous bone (< 17.7 weight % Ca) which reveals the percentage of bone area undergoing primary mineralization; CaHigh: the portion of bone areas with a calcium concentration >95th centile (> 25.3 weight % Ca) of the reference BMDD of cancellous bone (predominantly fully mineralized interstitial bone). The results were compared to those from young subjects with various forms of OI, including the two children with C-propeptide cleavage site mutations described by Lindahl *et al* [8].

Analysis of procollagen processing by dermal fibroblasts. Dermal fibroblasts were grown under standard conditions and then plated at subconfluent density in 35mm plates, and the proteins were labelled overnight with ^{3}[H]proline as previously described [19]. To assess the efficiency of cleavage of the carboxy-terminal propeptide, the cells were labelled in the presence of dextran sulfate to concentrate the protease and substrate to drive processing [20]. Proteins were separated on 5% SDS-polyacrylamide gels that contained 2M urea to enhance chain separation.

Bone collagen studies. *Bone preparation* Bone specimens were obtained from individuals A3 and F2. Control human bone was purchased from the Northwest Tissue Center, University of Washington, Seattle, WA. Bone was scraped clean and defatted with chloroform/methanol (3:1 v/v), and demineralized in 0.1M HCl, all steps at 4°C. Collagen was solubilized for SDS-PAGE by heat denaturation in SDS-PAGE sample buffer. Demineralized bone was also digested with bacterial collagenase as previously described

[21] and collagenase generated peptides were separated by reversed-phase HPLC (C8, Brownlee Aquapore RP-300, 4.6 mm x 25 cm) with a linear gradient of acetonitrile:n-propanol (3:1 v/v) in aqueous 0.1% (v/v) trifluoroacetic acid [22].

Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE). The chains of type I collagen extracted from bone were separated on 6% SDS-PAGE gels [23] and the portion of the gels that contained the separated α1(I) and α2(I) were cut from slabs and digested in-gel with Lys-C (rLys-C Mass Spec Grade, Promega, Madison WI) for mass spectral analysis.

Cross-link analysis. Bone was hydrolyzed in 6 N HCl, dried, dissolved in 1% (v/v) n-heptafluorobutyric acid and analyzed by C18 reverse-phase HPLC as described [24].

Mass spectrometry of bone peptides Electrospray MS was performed with Lys-C peptides and individual collagenase HPLC fractions using an LTQ XL ion-trap mass spectrometer (Thermo Scientific Waltham, Mass, USA) equipped with in-line liquid chromatography using a C4 5µm capillary column (300µm x 150mm; Higgins Analytical RS-15M3-W045) eluted at 4.5µl min. The LC mobile phase consisted of buffer A (0.1% formic acid in MilliQ water) and buffer B (0.1% formic acid in 3:1 acetonitrile:n-propanol v/v). The LC sample stream was introduced into the mass spectrometer by electrospray ionization (ESI) with a spray voltage of 4kV. Proteome Discoverer search software (Thermo Scientific) was used for peptide identification using the NCBI protein database.

Results

Phenotype variation. The median age-adjusted height for all individuals was on the 33th centile (range 1-90). About 80% of subjects had their first fracture by the age of 10, but only one had a fracture detected at birth. Fractures of the fingers and toes were particularly common, but one third had had a femoral or tibial fracture by the age of 25. Fractures continued into adulthood and included femoral (Fig 2A) and vertebral fractures (Fig 2B). In those over the age of 30, the total lifetime number of fractures ranged from 2 to 50. The frequency of fracture was lower after the age of 15. In adulthood, fracture rate varied considerably, but two-thirds of subjects still suffered fractures on average every 1 to 6

years. Fracture healing was normal, and resulted in long bone deformity in only one subject (A2). Of the 18 subjects over 15 years old, 6 (33%) had scoliosis; in one case (A2) this was severe. Two members of family F had scoliosis surgery aged 10 and 13, respectively.

Radiography showed dense bones in both the axial and appendicular skeletons. Unlike the usual forms of OI, the long bones were not narrow and had thick cortices with a scalloped appearance that developed with age (Figure 2C). Wormian bones were present in a minority of subjects in whom skull radiographs were available.

The sclerae were described as white in 67% and light blue or gray in 33%. Mild-to-moderate joint hypermobility was present in 77% of the 22 in whom this was assessed. All three members of family A had mild conductive hearing impairment (assessed by audiometry), but hearing impairment was not a feature in the other families. One subject (A2) had echocardiography-proven mitral regurgitation but cardiac murmurs were not detected in any of the other subjects.

Two members of family A suffered Achilles tendon rupture which healed with remarkable heterotopic calcification of the tendon (Fig 2D). Calcification within one patellar and one triceps tendon was also noted in patient A2. Three members of family C had tonsillar stones.

Three members of family F had a degree of enamel hypoplasia, but the teeth appeared normal in all the other individuals in whom dentition were explicitly described. Two adults from family F had suffered a fractured mandible during extraction of 3rd molar teeth. Both members of family B had delayed eruption of dentition. Fibro-osseous dysplasia of the jaw previously identified in B1 [9] was also seen in F2 (Fig 2E).

Biochemistry. Plasma P1CP concentrations were at or above the upper limit of normal ranging from 152 to 278 μg/l, (mean 229 [SD 56]; normal 70-165 μg/l), in contrast to the (expected) low concentrations in subjects with OI type I with haploinsufficiency mutations (mean 31 μg/l [SD 15], $p<0.0001$). Thus immunoreactive C-propeptide appeared in the circulation, despite the presumed disruption of the cleavage site.

Bone mineral density. Measurements were available in 22 subjects (14 female, 8 male). The mean lumbar spine BMD z-score was +2.9 [SD 2.1; range -0.8 to +7.7], and significantly greater than seen in the 22 subjects with OI type I due to *COL1A1* haploinsufficiency in whom the mean z-score was -2.2 [0.7; range -0.8 to -3.9] ($p<0.0001$; Figure 3). Seventeen subjects had paired lumbar spine and hip BMD. Although the mean z-score at the femoral neck was lower ($p<0.001$) than at the lumbar spine, it was still well above average: +2.6 [SD 1.8]. Spinal BMD z-scores were not correlated with age ($r=0.09$; $p = 0.72$).

The mean z-score at the lumbar spine was non-significantly higher for female (range -0.8 to +7.7; mean +3.5 [SD 2.3]) than male subjects (range +0.8 to +3.6, mean +1.6 [1.0]; $p=0.07$). Including data from the six patients reported in other papers [8,12,13] in the comparison, the mean z-scores were +3.1 (female) and +1.5 (male) ($p=0.14$).

The mean z-score at the lumbar spine was non-significantly higher in subjects with *COL1A1* mutations (mean +4.2 [SD 2.7]; range +1.2 to +7.7) than in subjects with *COL1A2* mutations (mean +2.4 [1.0]; range -0.8 to +5.3, $p=0.11$). Including data from six patients reported in other papers [8,12,13] in the comparison, the mean z-scores were +3.9 and +2.2, respectively ($p=0.16$).

Bone histology. A2, A3, G2 and G3 had transiliac bone biopsies, but only the latter two were suitable for quantitative histology (Table 2). The cortices were thick, with increased porosity, and the trabecular volume was also increased. There were focal areas of hyperosteoidosis in both trabecular bone and the Haversian systems, with increased osteoid seam width and osteoid up to 13 lamellae thick (Figure 4). The hyperosteoidosis appeared to be in two forms: either recently laid down osteoid, woven or lamellar in nature in association with osteoblasts, or in the form of unmineralised bone that in areas was overlaid by a thinner layer of mineralised bone. This raised the possibility that there is a period of delayed or intermittently reduced mineralisation. In support of this, the mineralization lag time was approximately three-fold greater than normal at 79-86 days. The bone appeared metabolically active with active osteoblasts in association with osteoid and surface osteoclasts readily seen.

Bone mineralization density distribution (BMDD). The distribution of the local mineral content in the bone matrix was measured on biopsy samples from A2 and A3. Both showed a shift toward higher mineralization in both cancellous and cortical compartments. Most remarkable was the increase in CaHigh which was from 12 to 16-fold greater than in normal adults. About 80% of the bone area was mineralized beyond the physiological plateau (25.3 weight % Ca) for normal bone matrix (Figure 5A).

Hypermineralization is a feature common to OI of various types. We compared the BMDD results (weighted cortical mean Ca-concentration) from adult subjects A2 and A3 with those obtained from bone samples from children with dominant mutations in *COL1A1* and *COL1A2* and a number of the recessive forms of osteogenesis imperfecta, including three children with *BMP1* mutations and the two children with C-propeptide cleavage site mutations described by Lindahl et al [8]. The cortical mean Ca-concentration was elevated in all the children to values close to the adult mean, but in A2 and A3 it was exceptionally high - far beyond the adult mean (Figure 5B).

Analysis of procollagen processing by cultured dermal fibroblasts. Dermal fibroblasts from A3 (*COL1A1* p.Ala1218Thr) synthesized and secreted type I and type III procollagens normally. Under the usual conditions of cell culture and labelling, the majority of type I and type III procollagen remain in the medium and are not efficiently processed. To determine if the C-propeptides of type I procollagen were efficiency cleaved, we labelled the cells in the presence of dextran sulfate, a volume excluder that concentrates the high molecular weight reagents and facilitated cleavage. Almost all the proteins made under these conditions are deposited in the cell layer. Under those conditions while the majority of proα1(I) chains were processed to α1(I) chains and deposited in the cell layer, a detectable amount was not processed and also seen in the cell layer (Fig. 6). No unprocessed chains were seen in the control cells.

Collagen processing in bone. In bone from one of the individuals with a *COL1A2* alteration (F2, p.Asp1120Asn) there was retention of a portion of the carboxyl-terminal propeptide of proα2(I) that contained the altered amino acid (aspargine, N). The peptides shown in red in Fig 7A have C-terminal residues that span the C-propeptidase cleavage site of the altered proα2(I) chain. Such peptides were completely absent from control human bone samples

and from the bone of subject A2 with a *COL1A1* mutation. The latter also gave no comparable cleavage site spanning peptides from proα1(I) chains, only those from the correctly cleaved C-terminus (Tyr-Tyr-Arg-Ala) or a trimmed form (Tyr-Tyr-Arg). Though not shown, all collagenase digests of demineralized bone also revealed low levels of peptides that had derived from the C-propeptide globular domain (of both proα1(I) and proα2(I) chains). This was true for all mutant and normal bone samples but quantitative comparison of yields was not possible.

To determine if the collagen that was incorporated into the mature bone matrix contained the abnormal molecules we examined the peptides derived from collagens extracted from bone with a denaturing reagent (Figure 7B). The mobility of the chains on SDS-PAGE showed no signs of this or of longer precursor forms. On digestion with the proteinase endo-Lys C, the recovered C-terminal peptide from the excised α2(I) chains showed only the correct propeptidase-cleaved terminus, plus shorter trimmed ends normally seen in digests of bone collagen. The source of the extended peptides shown in Fig 7A does not therefore appear to be matrix collagen molecules with retained extensions of a similar size.

Discussion

The phenotype of subjects with missense mutations at the C-propeptide cleavage site is generally mild and similar to that of OI type I that results from haploinsufficiency in terms of stature, fractures, hypermobility, deformity, scoliosis and the absence of dentinogenesis imperfecta. There are, however, major differences: in most cases the sclerae are not blue, deafness is uncommon, and there is a striking increase in bone mass, with thick cortices in long bones and osteosclerosis of the axial skeleton in many cases. Deformity was present in only one subject, and not described in other reports, apart from one child in the family with the same *COL1A2* mutation as family F, described by Nishimura et al [10]. Other features noted in some families included heterotopic calcification in tendons that had suffered injury and fibro-osseous dysplasia of the jaw. The association of fibro-osseous dysplasia or cementoma of the jaw with OI has been noted previously [25,26].

The impression of high bone mass is borne out by bone densitometry studies, with median z-scores of +3 relative to the normal population (markedly higher than in people with OI type I), and the exceptionally high bone matrix mineralization as indicated by the BMDD analysis in two adult patients. In the two pediatric cases reported by Lindahl *et al* [8] the BMDD measurements, though higher than in normal children, were similar to the findings in children with other types of OI. However, we found no correlation between BMD z-scores and age, and in Lindahl's study BMD z-scores were unchanged in sequential studies in a girl between the ages of 8 and 12½ years [8]. Despite the high bone mass found on densitometry, and hypermineralization seen on BMDD, hyperosteoidosis was found on bone histology, as it was in the cases studied by Lindahl et al [8]. Dynamic histomorphometry, based on tetracycline-labelling, showed that the mineralization lag time was prolonged, suggesting a paradoxical delay in mineralization, though this was not to the degree seen in classical osteomalacia (>100 days).

The clinical presentations appeared similar whether the mutation changed either the alanine or aspartate at the cleavage site, or whether the C*OL1A1* or *COL1A2* gene was affected. The BMD values were somewhat higher in female compared to male subjects. In families where there were siblings or cousins of comparable age but different gender (five comparisons in four families) the male family members had suffered more fractures, and earlier in life, in four comparisons, with no difference in one. However, the number of subjects is too small to be definitive on these points. We found that BMD was greater at the spine than the hip, but in two post-menopausal women described by Rolvien et al [13] the hip BMD was substantially higher than the at the lumbar spine. In the report of Le Quesne Stabej et al [12] one man who was mosaic for the same *COL1A1* mutation as families A and B, also had a high spinal BMD (z-score +3.6).

The C-propeptide of procollagen I is cleaved by a number of tolloid-like proteinases, the most important of which is bone morphogenetic protein 1 (BMP1) [27], but other proteinases, such as mammalian tolloid (mTld, an alternatively-spliced form of BMP1) and two closely related proteinases, mammalian tolloid-like 1 and 2, can also cleave the C-propeptide. All appear to cleave at the same di-peptide on both chains [28,29]. We were

able to detect P1CP in the circulation indicating that cleavage of the C-propeptide was occurring.

Patients with inactivating mutations in *BMP1* have decreased protein levels of both the alternatively spliced gene products and impaired cleavage of the C-propeptide. Asharani *et al* found detectable plasma P1CP at levels just below the normal range in two patients with mutations that affected the signal peptide of BMP1 [30], which confirmed that cleavage of the C-propeptide occurred; suggesting that alternate enzymes facilitated the event. To date 18 people with OI due to *BMP1* mutations have been described [30-37]. They share some phenotypic features with those with substrate alterations– white sclerae and absence of dentinogenesis imperfecta – but their fracture phenotype is more severe: 28% had fractures *in utero* or at birth and 85% had fractured by the age of 2 years. High bone density has been found in some, but not all, patients with *BMP1* mutations [30,31,32,37]. Bone histology and BMDD analysis of biopsies from two subjects with *BMP1* mutations and high BMD showed the same paradoxical combination of hyperosteiodosis and hypermineralization [30,33], as we observed in subjects with C-propeptide cleavage site mutations. Hoyer-Kuhn et al [38] suggested that mineralization might be affected in two ways: a delay in onset initiation and then incorporation of too much mineral into the matrix.

The activity of BMP1 is potentiated by a secreted glycoprotein, PCOLCE1, that binds specifically to the type I procollagen C-propeptide and to the C-telopeptide (linking the C propeptide to the triple-helical domain), and seems to act by inducing a conformational change that renders procollagen a fitter substrate for C-propeptide cleavage. Of note, the *pcolce1* knock-out mouse has impaired cleavage of the C-propeptide and high bone mass, but the bone has inferior material properties [39].

While the high bone mass phenotype associated with mutations at the C-propeptide cleavage site is distinctive, the mechanism underlying the hypermineralization remains unknown. Type I collagen molecules (from which the N- and C-propeptides have been cleaved) assemble into fibrils in which the molecules have a characteristic array that forms a scaffold for the organised arrangement of uniaxially-orientated apatite crystals. The extracellular assembly of collagen molecules (~300 nm in length) creates so-called ‘gap’ and ‘overlap’ zones (Fig 8). Arrays of collagen maintain precise registration of their gap and

overlap regions, so that channels are created through the array. The maintenance of a net positive charge close to the C-terminal end of the collagen molecules is thought to promote infiltration of amorphous calcium phosphate [40,41] into the 40nm long gap zone, and clusters of charged amino acids, both in the gap and overlap regions form nucleation sites and control the conversion of amorphous calcium phosphate into apatite crystals. Once nucleated in the gap region the apatite crystals then grow in parallel to each other and to the collagen molecules associated with the crystals. Interfibrillar collagen mineralization (along and on type I collagen fibril surfaces and within regions between collagen fibrils) is less well understood but may involve interaction with proteins of the SIBLING family of non-collagenous acidic proteins (including bone sialoprotein, dentin matrix protein 1, dentin sialophosphoprotein, osteopontin and matrix extracellular phosphoglycoprotein) [42]. They can bind to collagen surfaces where their charged or phosphorylated conformations extend into interfibrillar spaces, attract calcium and phosphate ions, and lead to nucleation. Such interactions are thought to contribute to the deposition of mineral between collagen fibrils and fibers in extracellular tissue spaces and modulate the rate of crystallization within the bone collagen fibril matrix [40, 41, 43].

How mutations at the C-propeptide cleavage site or in the genes that encode the cleavage enzymes might affect any of these processes is yet to be elucidated. One possibility is that retention of short extended peptides facilitates mineral deposition in collagen. The occurrence of tendon calcification, tonsillar stones and fibro-osseous dysplasia of the jaw in some families suggest a generalized tendency to hypermineralization. Alternatively, secondary cellular effects of delayed collagen processing on regulatory pathways of mineral deposition may be responsible. Studies of mineral particle characteristics of bone from OI type I patients using small-angle x-ray scattering and wide-angle x-ray diffraction indicate that the higher BMDD in OI is due to greater packing density rather than particle size [44].

The high-normal concentrations of C-propeptide in the circulation is consistent with the studies in dermal fibroblasts and decalcified bone indicating that there was retention of the propeptide on only a fraction of the collagen in the presence of mutations at the Ala-Asp cleavage site. The mass spectometric analyses of extracts of bone collagen from a patient with a *COL1A2* mutation revealed short peptides that spanned the C-propeptidase cleavage

site in of the proα2(I) chain that were not found in control bone (Fig 7A). The lack of similar peptides from the proα1(I) chain from bone of a subject with a *COL1A1* mutation may reflect either sequence differences C-terminal to the C-propeptidase cleavage site that prevented informative peptides from being generated from proα1(I) by bacterial collagenase digestion, or efficient cleavage proximal to the site.

Since whole decalcified bone was digested it is uncertain whether the cleavage-site spanning peptides from *COL1A2* mutant bone were derived from matrix collagen or a cell-associated pool of procollagen. The lack of cleavage-site spanning peptides from an in-gel digest of denaturant-extracted α2(I) chains (Fig 7B) suggests that they may have originated from an accumulation of intracellular or recently made procollagen that had so far escaped intracellular degradation or processing by alternative proteases. An alternative explanation is that there is preferential incorporation of the normal molecules whilst the abnormal ones might have been extracted by the non-denaturing solvents.

The cause of the high bone/hypermineralization mass phenotype remains unknown, but clarification of its pathogenesis could come from better understanding of the cleavage process (such as alternative cleavage sites and the roles of PCOLCE1 and proteinases other than BMP1) and x-ray diffraction studies to determine if mineral packing density is exaggerated.

Bisphosphonates are commonly used in children with osteogenesis imperfecta [45], with an improvement in BMD commonly used as a surrogate measure of their effectiveness. The paradoxical associations of impaired mineralization, high bone mass and fragility make problematic the decision whether to give bisphosphonate treatment, since it is generally contraindicated in the presence of mineralization defects and/or when bone mass is high. After the evaluations described here, 2 adults and 1 child were treated with bisphosphonates to try to reduce the rate of fracture, but the data are insufficient to draw any conclusion as to its effectiveness. On a cautionary note, an older patient with a *COL1A2* C-propeptide cleavage site mutation who was treated with a bisphosphonate developed osteonecrosis of the jaw [13], and in a child with *BMP1* mutations bisphosphonate therapy was associated with an apparent increase in fracture rate [37].

We found that missense mutations affecting the C-propeptide cleavage site are associated with a highly distinctive OI phenotype of mild to moderate severity, characterized by high bone mass. In terms of fracture, the phenotype is not as severe as that seen in people with *BMP1* mutations, but the bone density is, on average, higher. As with autosomal dominant osteopetrosis [46], this syndrome emphasise that high bone mass does not necessarily translate into a low fracture risk. The total number of people identified with such mutations is still small, so the full breadth of the phenotype remains to be determined.

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Table 1 Demographic details of the subjects involved and mutations found in each family

Family	Origin	Gene	Mutation	Effect	Subject	Gender	Age
A	New Zealand	*COL1A1*	c.3652G>A,	p.Ala1218Thr	A1	M	75
					A2	M	41
					A3	F	38
B	Australia	*COL1A1*	c.3652G>A	p.Ala1218Thr	B1	F	28
					B2	M	5½
C	USA	*COL1A1*	c.3652G>C	p.Ala1218Pro	C1	F	3½
D	USA	*COL1A1*	c.3655G>A	p.Asp1219Asn	D1	F	22
					D2	F	3½
E	UK	*COL1A1*	c.3657T>G	p.Asp1219Glu	E1	M	49
					E2	M	45
					E3	F	19
					E4	M	13
F	New Zealand and UK	*COL1A2*	c.3358G>A	p.Asp1120Asn	F1	F	72
					F2	M	41
					F3	F	39
					F4	F	7
					F5	M	6
					F6	F	30
					F7	F	26
					F8	M	41
					F9	M	6
					F10	M	3
G	Denmark	*COL1A2*	c.3358 G>C	p.Asp1120His	G1	F	74
					G2	F	49
					G3	F	34
H	Canada (Iraqi)	*COL1A2*	c.3359 A>C	p.Asp1120Ala	H1	M	51
					H2	F	12½
					H3	F	10½
					H4	M	10½

Table 2 Quantitative histomorphometry findings in two subjects

Subjects (gender, age)	**G3 – F 49 yo**	**G2 – F 34 yo**	**Normal range (Adult)**
Indices of cortical bone mass (outer cortex)			
Mean cortical width (um) - Ct.Wi	3334	2899	909 +/- 98
Cortical porosity (%) - Ct.po	11.8	14.1	6.3 +/- 0.6
Indices of trabecular bone mass			
Trabecular bone volume (%) - BV/TV	54.8	42.6	22.5 +/- 3.5
Trabecular width (um) - Tb.Th	521	255	213 +/- 65
Indices of osteoid tissue			
Trabecular osteoid surface (%) - OS/BS	19.6	17.3	19.3 +/- 3.0
Osteoid seam width (um) - O.Th	28.9	22.7	9.5 +/- 0.6
Trabecular osteoid volume (%) - OV/BV	2.4	1.2	1.9 +/- 0.4
Trabecular osteoblastic osteoid (%) - Ob.S/BS	2.8	2.1	1.3 +/- 0.5
Indices of resorption			
Trabecular eroded surface (%) - ES/BS	1.4	1.5	5.1 +/- 0.6
Osteoclastic resorptive surface (%) - Oc.S/BS	0	0	0.13 +/- 0.06
Indices of bone formation (Tetracycline-derived)			
Mineral apposition rate (um/d) - MAR	1.10	0.69	0.64 +/- 0.01
Mineralization lag time (days)	79	86	29 +/- 3

Legends to Figures

Figure 1. Amino acid changes at the C-propeptide cleavage site in families A to H and other reported cases. In both *COL1A1* and *COL1A2*, cleavage takes place between an alanine (A) and an aspartate (D) residue, as shown by the dashed line. The p.Asp1219Asn mutation in *COL1A1* in family D has previously been reported in one additional individual [8], and the p.Asp1120Asn mutation in *COL1A2* in family F was also found in a Japanese family [10]. Lindahl *et al* [8] also reported the p.Aln1119Thr mutation in *COL1A2* in one additional individual; Rolvien et al [13] reported the p.Asp1120Gly mutation.

Figure 2A Femoral fracture in a 35 year-old man with a p.Asp1219Glu mutation in *COL1A1*. Note the thick femoral cortices.

Figure 2B Vertebral fractures (indicated by arrows) that presented as postpartum osteoporosis in a 38 year old woman from family F with a p.Asp1120Asn mutation in *COL1A2*.

Figure 2C Radiographs of the elbow in a man (individual A1) with a p.Ala1218Thr mutation in *COL1A1* taken at ages 41 and 72 years. Note the new fracture and the thick cortices that have become scalloped with age.

Figure 2D Tendon ossification that occurred after rupture of the Achilles tendon in a father (left) and adult son (right) from family A with a p.Ala1218Thr mutation in *COL1A1*.

Figure 2E Fibro-osseous dysplasia of the mandible (indicated by arrows) in a man with a p.Asp1120Asn mutation in *COL1A2*. Similar findings were reported in an indidual with n p.Ala1218Thr mutation in *COL1A1* [9].

Figure 3 Lumbar spine bone mineral density (BMD) in 21 subjects with C-propeptide cleavage site missense mutations compared to 23 subjects with haploinsufficiency mutations (OI type I), separated according to gender.

Figure 4 Histological findings on transiliac bone biopsy (undecalcified bone).
Upper panel: biopsy from an individual from family F with a p.Asp1120His mutation in *COL1A2*. The outer cortex (to the left of the picture, arrowed) is 3 times thicker than average and the trabecular volume twice the average for age. Lower panel: hyperosteoidosis (orange colour) with increased osteoid thickness (up to 13 lamellae visible under polarised light) in Haversian canals of a patient with a p.Ala1218Thr mutation in *COL1A1* from family A (Goldner trichrome stain).

Figure 5A Bone mineralization density distribution (BMDD) histograms derived from qBEI-images in biopsies from a 41 year-old man and a 39 year old woman with the p.Ala1218Thr

mutation in *COL1A1* (from family A). The dotted line indicates the cancellous bone compartment and the solid line the cortical compartment. There is a marked shift to higher mineralization: Approximately 80% of the bone area was mineralized beyond the normal physiological plateau level of 25.3 weight % Ca as indicated by the BMDD parameter CaHigh.

Figure 5B Comparison of BMDD from cortical bone in the two adults whose data are shown in 5A with the results from children with various types of osteogenesis imperfecta due to mutations in the genes indicated. The mean value and range for each type of OI, and the normal mean values for unaffected children and adults are indicated. The values in the adult patients with a *COL1A1* mutation substantially exceed those of children with *BMP1* mutations and the two children with C-propeptide mutation cleavage site mutations described by Lindahl et al [8] (shaded box).

Figure 6 Skin fibroblast studies. Cells from the controls and a patient with a p.Aal1218Thr mutation in *COL1A1* were incubated overnight either without or in the presence of dextran sulfate and radiolabeled proline. The proteins from the medium from the untreated cells and the cell layer from the treated cells were harvested and separated by SDS-polyacrylamide gel electrophoresis under reducing conditions. Dextran sulphate concentrates by volume exclusion so that large molecules are in much higher concentration outside the gel that is formed and procollagen proteins that clear at both ends interact more efficiently. In the cell layer from the affected subject's cells there is accumulation of pCα1(I) chains that have not been cleaved by BMP1.

Figure 7A

Mass spectroscopic analysis of a total bacterial collagenase digest of decalcified bone collagen from a subject with a p.Asp1120Asn mutation in *COL1A2*. The peptides with red C-terminal residues span the C-propeptidase cleavage site in the α2(I) chain. Such peptides were completely absent from all control human bone and from bone from an individual with an p.Ala1218Thr mutation in *COL1A1* mutant bone The latter gave no comparable cleavage site spanning peptides from α1(I), only the correct cleaved C-terminus FYRA or the trimmed FYR. Though not shown, the same collagenase digests reveal internal C-propeptide fragments from all bone digests (controls and in both mutants).

Figure 7B

Results of analysis to determine if the α2(I) chains of matrix type I collagen had any extensions. The mobility of the chains on SDS-PAGE showed no signs of extensions, or of longer pro-forms in denatured extracts of the decalcified bone. Using the proteinase endo-Lys C, the C-terminal peptide from the excised α2(I) chain showed only the correct propeptidase cleaved terminus plus further trimmed ends normally seen in digests of bone collagen.

Figure 8

A depiction of intrafibrillar collagen mineralization showing the 2D and 3D organization, assembly, and initial mineralization of collagen molecules. (a) A single collagen molecule, 1.23 nm in diameter and 300 nm in length. (b) Collagen molecules are then assembled into a 2D aggregate, cross-linked into a quarter-staggered array with characteristic hole (40 nm) and overlap (27 nm) zones. Packing of consecutive 2D arrays into 3D assemblages occurs with strict registration of all hole and overlap zones so that channels are created. (c) These channels are the principal sites in which nucleation of mineral crystals occurs. (d) Once nucleated in the collagen hole zones, the crystals, shown in blue, grow preferentially in their c-crystal axis direction. The pore spaces (0.24 nm) between adjacent collagen molecules constitute narrow regions whose capacity to accommodate mineral is unknown. Adapted, with permission, from Landis & Jacquet [41].