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Mutations in type I collagen genes resulting in osteogenesis imperfecta in humans✪

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Osteogenesis imperfecta (OI), commonly known as "brittle bone disease", is a dominant autosomal disorder characterized by bone fragility and abnormalities of connective tissue. Biochemical and molecular genetic studies have shown that the vast majority of affected individuals have mutations in either the *COL1A1* or *COL1A2* genes that encode the chains of type I procollagen. OI is associated with a wide spectrum of phenotypes varying from mild to severe and lethal conditions. The mild forms are usually caused by mutations which inactivate one allele of *COL1A1* gene and result in a reduced amount of normal type I collagen, while the severe and lethal forms result from dominant negative mutations in *COL1A1* or *COL1A2* which produce structural defects in the collagen molecule. The most common mutations are substitutions of glycine residues, which are crucial to formation and function of the collagen triple helix, by larger amino acids. Although type I collagen is the major structural protein of both bone and skin, the mutations in type I collagen genes cause a bone disease. Some reports showed that the mutant collagen can be expressed differently in bone and in skin. Since most mutations identified in OI are dominant negative, the gene therapy requires a fundamentally different approach from that used for genetic-recessive disorders. The antisense therapy, by reducing the expression of mutant genes, is able to change a structural mutation into a null mutation, and thus convert severe forms of the disease into mild OI type I.

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Abbreviation: OI, osteogenesis imperfecta.

OSTEOGENESIS IMPERFECTA — "BRITTLE BONE DISEASE"

Osteogenesis imperfecta (OI) is a heterogeneous genetic disorder of connective tissue, commonly known as "brittle bone disease". The major clinical feature that defines OI is bone fragility. Fractures may be rare or frequent (more than 200 prior to puberty) and bone fragility may be accompanied with reduced life span and various connective tissue abnormalities. On the basis of clinical, radiologic and genetic criteria, Sillence *et al.* (1979) identified four types of OI (Table 1). The mildest form is OI type I characterized by blue sclerae, premature deafness, and mild to moderate bone fragility. It is subclassified as IA if the teeth are normal, or IB if there is dentinogenesis imperfecta. The most severe form is the lethal perinatal form, OI II. Infants with type II OI experience intrauterine fractures, intracranial hemorrhage after vaginal delivery, and succumb to death shortly after birth. The children with type III OI may die in infancy of respiratory problems, and many others will die in their childhood from pneumonia, cor pulmonale, or trauma such as skull fracture. Those infants who survive suffer from gradual deformity of the long bones and spine due to fractures; they require multiple orthopedic rodding procedures and wheelchairs for mobility. Type IV OI is the least common and has some similarities to mild type I and type III phenotypes. In childhood, there is usually moderate bowing of long bones, and the fracture rate decreases around puberty. Many affected individuals have moderately short stature, osteoporosis and mild scoliosis. Recently, two groups of patients initially classified as OI type IV, turned out to have unique clinical and histological features. These new forms have subsequently been named types V (Glorieux *et al.*, 2000) and VI (Ward *et al.*, 2000). OI affects probably more than one per 10000 individuals; some persons, however, are not diagnosed until later on in their lives.

Biochemical and molecular genetic studies have shown that the vast majority of individuals (>90%) affected with OI types I–IV, have mutations in either the *COL1A1* or *COL1A2* genes that encode the chains of type I procollagen, the major structural protein of bone, skin and tendons. The genetic defect underlying OI types V and VI remains to be elucidated, as it does not appear to be associated with collagen type I mutations. In many cases, OI is inherited in an autosomal dominant manner but new mutations are frequent. A few cases result from autosomal recessive inheritance. Some patients suffer from OI as a result of mosaicism in a parent who shows little or no clinical abnormality (Zlotogora, 1998). Since so far osteogenesis imperfecta has been an incurable genetic disease, cell therapy and gene therapy are being investigated as potential treatments.

Table 1. Classification of OI phenotype according to Sillence *et al.* (1979)

OI type	Inheritance	Clinical characteristics
I	AD	Normal stature, little or no deformity, blue sclerae, hearing loss
II	AD (new mutations)	Lethal in perinatal period, beaded ribs, long bone fractures
III	AD AR (rare)	Progressively deforming, short stature, multilpe fractures, triangular facies, hearing loss
IV	AD	Moderately severe, variable short stature, dentinogenesis imperfecta, osteoporosis, bowing of long bones

AD, autosomal dominant; AR, autosomal recessive.

THE TYPES OF MUTATIONS IDENTIFIED IN OSTEOGENESIS IMPERFECTA

Mutations in type I collagen genes resulting in OI can be considered in two major categories, mutations that resulted in exclusion of the product of the mutant allele from the mature collagen molecule, and those which permitted the incorporation of a structurally abnormal chain, referred to by Sykes (1985) as "excluded" and "included" mutations, respectively. In the first group, lack of expression of mutant product usually results from heterozygosity for premature termination codons in the *COL1A1* gene (Willing *et al.*, 1996). The "included mutations" result in the generation of abnormal type I procollagen molecules. The triple helical portion of either collagen chain contains 338 uninterrupted repeats of the triplet GXY, where G is glycine, X is often proline, and Y is often hydroxyproline. The presence of glycine, which has the smallest side chain in every third residue, is a prerequisite for correct folding of the three α chains into a collagen triple helix. The great majority of mutations appear to result in substitutions for glycine residues within the triple helix or if mutations occur in the consensus splice donor or acceptor sites, are the product of exon skipping events (Cole, 1994; Dalgleish, 1998). Multiexon rearrangements, shorter deletions, insertions or frameshift mutations are rare.

EFFECT OF MUTATIONS ON THE EXPRESSION OF TYPE I COLLAGEN

A description of type I collagen genes and the manner in which the collagen is synthesized and processed by the cell might help in understanding how mutations in these genes result in OI. Two genes, *COL1A1* located on chromosome 17, and *COL1A2* located on chromosome 7, encode the proα1(I) and proα2(I) chains of type I procollagen, respectively (Kielty *et al.*, 1993). These genes are composed of about 50 exons scattered over 18 kb (for α1(I)) and 38 kb (for α2(I)) of chromosomal material. Most of the exons consist of 54 or 108 base pairs, and the final coding mRNAs in the cytoplasm range in size from 5.5 to 7.2 kb. Fibril-forming collagens such as type I collagen are synthesized into larger precursors, known as procollagens, which contain globular N-terminal and C-terminal propeptides. Two proα1 and a single proα2 chains first associate by hydrophobic and electrostatic interactions among the C-propeptides (Fig. 1A). The association is then stabilized by the formation of interchain disulphide bonds. From the carboxyl end, helix formation proceeds towards the amino terminal end in a zipper-like fashion. Simultaneously, proline and lysine residues are hydroxylated by specific hydroxylases and hydroxylysine residues are glycosylated by sugar transferases. These modifications occur only in chains that are in nonhelical configuration and cease as soon as the protein folds into a triple helix. In the extracellular space, specific N- and C-peptidases cleave the terminal extensions. The mature helical molecules self-aggregate into highly ordered fibrils stabilized by intermolecular cross-links formed by oxidative deamination of lysine and hydroxylysine residues. Mechanical strength of connective tissue is due mainly to fibrils which form a template for matrix deposition. In bone, the fibrils are the template for mineralization, i.e., in this case, for incorporation of hydroxyapatite crystals. The mechanical properties of bone are dependent on an intimate association between the collagen fibrils and crystals of hydroxyapatite.

The "excluded mutations" usually result in one null *COL1A1* allele and, although only half of the normal amount of type I procollagen becomes synthesized, its structure is normal. The remaining mutations, which alter the primary structure of collagen, affect the protein in several ways. Almost all molecules that contain chains with mutations

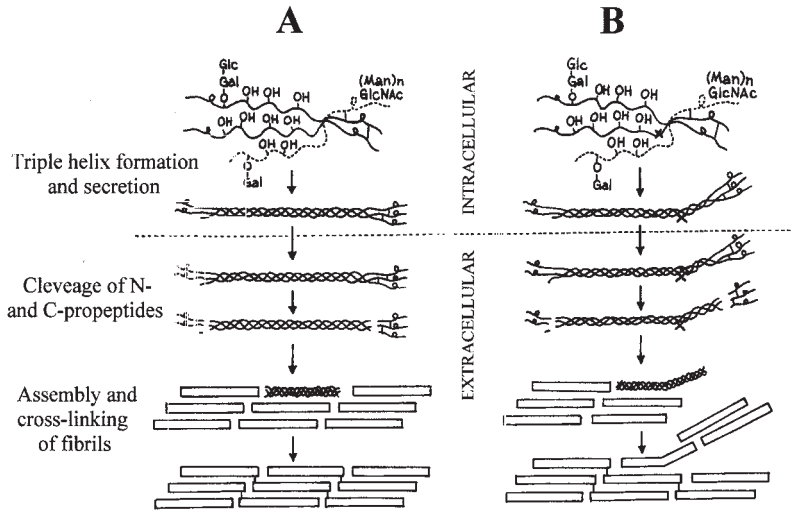

Figure 1A. Schematic representation of the intracellular and extracellular steps involved in the synthesis, processing, and assembly of type I collagen molecules into fibrils. B. Mutation which produces a conformational change such as a kink leading to the defective fibril formation.

The sign (×) in the procollagen chain indicates the point mutation.

in the triple-helical domain, are less stable than their normal counterparts (Baker *et al.*, 1989). Another consistent observation is the overmodification of the portions of the α-chains N-terminal to the mutation (Lehmann *et al.*, 1995) as judged by the lower electrophoretic mobility of the collagen chains on SDS/PAGE (Fig. 2). Since post-translational modification of pro α chains occurs only in the unfolded state, the unidirectional overmodification of lysyl and hydroxylysyl residues N-terminal to the point mutation (as opposed to the normal modification C-terminal to the mutated site) has been interpreted as a result of delayed folding N-terminal from the site of the mutations (Raughunath *et al.*, 1994). The mutations which occur within the domains that encode the carboxyl-terminal propeptide may alter the ability of chains to aggregate into molecules (Chessler *et al.*, 1993). Some substitutions for obligate glycines interrupt the zipper-like folding of the triple helix and generate unfolded procollagen that first accumulates in fibroblasts and then is degraded. The effects of the mutations are amplified because both the normal and mutated chains present in the same molecule are degraded in a process re-

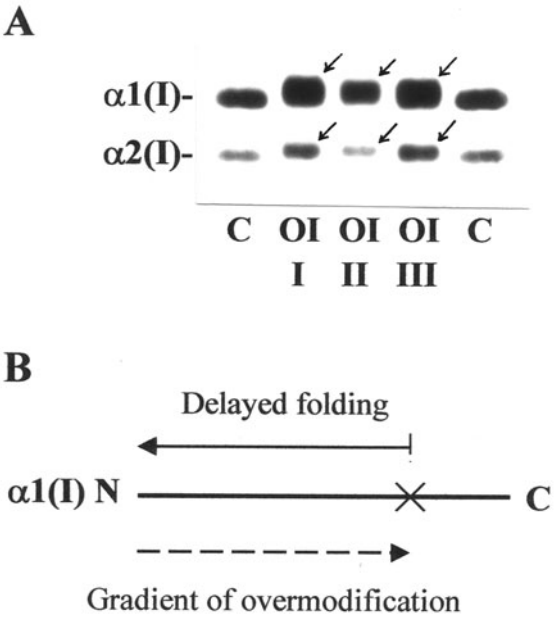

Figure 2A. SDS/PAGE of type I collagen secreted by fibroblasts from three infants with lethal type of disease (OI I, OI II, and OI III, respectively) and a control (C).

Cells were incubated with [^{3}H]proline overnight in the presence of ascorbic acid. Arrows indicate the overmodified forms of the α1(I) and α2(I) chains present in the OI media.

B. Helix formation from carboxyl (C) to amino (N) ends (delayed from the point mutation) and gradient of overmodification (in opposite direction) due to the point mutation (×) in the collagen chain.

ferred to as "procollagen suicide" or a dominant negative effect (Prockop & Kivirikko, 1984). Other consequences of mutations include defective processing of extracellular type I procollagen by N-proteinase (Vogel *et al.*, 1988) or increased susceptibility of trimers to proteases (Valli *et al.*, 1993). Studies on fibril formation demonstrated that molecules with such a mutation that introduced a flexible kink into the triple helix, copolymerized with the normal molecules (Fig. 1B). The presence of the kinked molecules delayed fibril formation, reduced the total amount of collagen incorporated into the fibrils, and drastically altered the morphology of the fibrils (Vogel *et al.*, 1988). Since the integrity, architecture and functions of connective tissues are the result of specific interactions between collagen, proteoglycans and structural glycoproteins, the presence of abnormal collagen chains may have a strong influence on the metabolism of noncollagenous components (Tenni *et al.*, 1988).

TRANSLATION OF MUTATION TO PHENOTYPE

The phenotypic consequences of mutations in type I collagen genes reflect the type of mutation, gene in which the mutation occurred and location of the mutation. Mutations in the *COL1A1* gene resulting in the synthesis of half the normal amount of functional proα1(I) chains (because of one null allele) have but mild clinical consequences found in individuals with type I OI (Fig. 3). Normal type I procollagen molecules require a minimum of two proα1(I) chains for stability and when the number of chains available is halved, the amount of type I procollagen produced is diminished to 50% of normal. The phenotypic effects of "included mutations" that result in the generation of abnormal type I procollagen molecules are more deleterious than those of null mutations. If an abnormal chain leads to very rapid intracellular degradation of molecules that incorporate the chain, the clinical

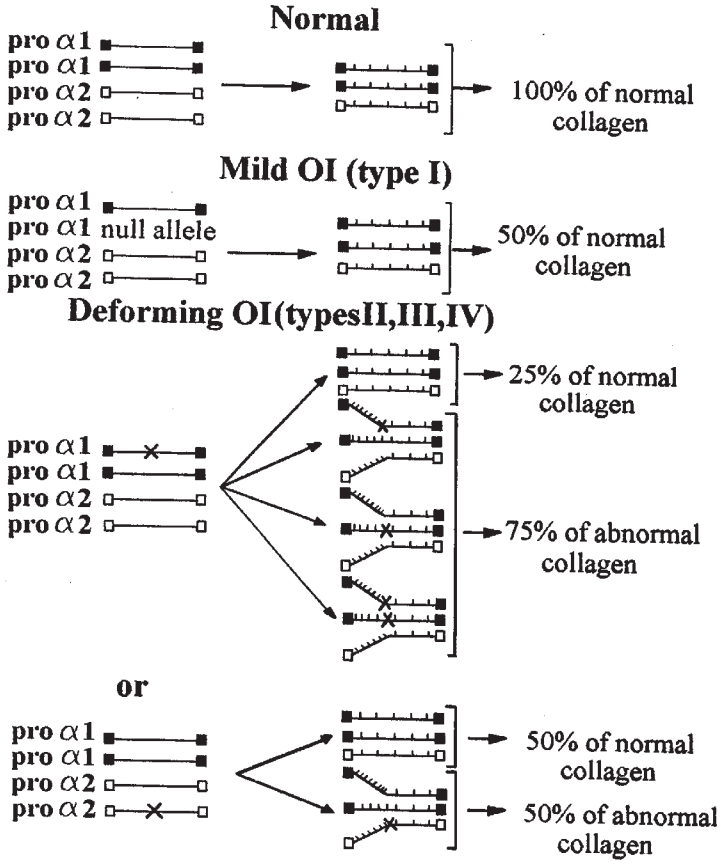

Figure 3. The molecular mechanism of OI.

Mutations which decrease the synthesis of normal type I collagen to 50% (because of one null allele of *COL1A1* gene) result in mild type I OI. The mutations that affect the structure of the procollagen chains have more deleterious phenotypic consequences (types II, III and IV OI). The gene, in which the mutation occurs, affects the proportion of abnormal molecules and is probably reflected in the phenotype. The signs (×) indicate mutations in the procollagen chains; the small vertical lines indicate the posttranslational modifications, increasing from the amino end to the point mutation.

consequences would differ, depending on the gene in which the mutation did occur. Mutations in the *COL1A1* gene may be highly deleterious, and even lethal, because they comprise three-quarters of all the procollagen molecules of type I synthesized (Fig. 3). In contrast, similar mutations in the *COL1A2* gene would result in the loss of only half the molecules made and so might be similar in effect to a null *COL1A1* allele.

It has also been proposed that the position of a mutation along the chains may be a crucial factor, with mutations closer to the carboxyl end resulting in a more severe phenotype than mutations near the amino terminal end of the chain (Starman *et al.*, 1989). This gradient is modified by the nature of the substituting amino acid, so that some of them may be lethal when incorporated at any place along the entire domain (e.g., aspartic acid) while others may show a lethal to a nonlethal transition in the carboxyl terminal half of the chain (e.g., cysteine). Such gradients are not apparent in the case of other substitutions although domains producing lethal and nonlethal phenotypes have been proposed to exist in the α2(I) chain (Marini *et al.*, 1993). Functions associated with crucial *versus* noncrucial domains might include their role in thermal stability of collagen, its secretion from the cell or interactions with collagenous or noncollagenous molecules in the extracellular matrix. The genetic background and other modifiers appear to be important, as the same mutation has been observed to result in different phenotypes (Zhuang *et al.*, 1996). We have found that defects in collagen metabolism (produced by substitution of arginine for glycine at position 388 in the α1(I) chain (unpublished) are correlated with a decrease in the activity of prolidase, an enzyme essential for collagen synthesis and cell growth. This observation might point to another an important mechanism being involved in producing the OI phenotype (Galicka *et al.*, 2001).

WHY MUTATIONS IN TYPE I COLLAGEN GENES CAUSE BONE DISEASE?

The type I procollagen is the major structural protein of skin, bone and tendons. So far, most studies have been focused on procollagen synthesized by fibroblasts cultured from skin biopsies of OI patients. Genomic mutations are presumably expressed in all type I collagen-producing tissues, but OI primarily affects the skeletal system. Therefore, to study the mechanism through which collagen mutations lead to the clinical phenotype of OI, the most appropriate cells are osteoblasts, as they are of greatest significance for skeletal pathophysiology. Some reports suggested that the patient's osteoblasts expressed type I collagen in a different way than did fibroblasts. In patients with type IV OI, the osteoblasts contained a higher proportion of mutant collagen as compared to the fibroblasts (Sarafova *et al.*, 1998; Galicka *et al.*, 2002). We found that the mutant collagen was secreted more rapidly by bone than by skin cells (Galicka *et al.*, 2002). Furthermore, the osteoblast mutant collagen molecules showed higher thermal stability (Sarafova *et al.*, 1998; Galicka *et al.*, 2002). The presence of mutant collagen in bone, but not in skin matrix, suggests that the incorporation of abnormal molecules is tissue specific (Mundlos *et al.*, 1996). It has been shown that mutant molecules are incorporated into the extracellular matrix of bone efficiently and represent a high percentage of total collagen isolated from whole cortical bone (Niyibizi *et al.*, 1992). The copolymerization of normal and mutated molecules which induced formation of poor and disorganized matrix, had a dramatic effect on mineral deposition (Cohen-Solal *et al.*, 1994).

While type I collagen is the major organic component of both skin and bone, there are many differences between these tissues in the composition of other matrix components. The

noncollagenous components of bone, involved in matrix organization and mineralization, include: osteonectin, which acts as a nucleator in collagen mediated mineralization, decorin, which is important in fibril formation, the large chondroitin sulfate proteoglycan and hyaluronan, which can "capture space" for subsequent matrix deposition, as well as biglycan associated with osteoblast differentiation (Scott, 1988). Studies on bone matrix of OI patients showed not only reduced levels of some components (collagen, osteonectin, the large chondroitin sulfate proteoglycan, biglycan, and decorin) in bone cells, but also elevation of some other matrix components (thrombospondin, fibronectin, and hyaluronan) (Fedarko *et al.*, 1995). These quantitative changes of matrix components in OI bone, in addition to the mutant collagen, may play a significant role in OI bone pathology. Finally, the bone requirements for molecular structure may be greater than those of skin and other soft tissues, and this might explain, in part, the relative tissue specificity of the mutations discussed.

APPROACHES TO THE GENE THERAPY OF OSTEOGENESIS IMPERFECTA

The experiments with expression of collagen genes with mutations in transgenic mice directly confirmed the conclusion that the mutations in the genes for type I procollagen lead to the OI phenotype (Forlino *et al.*, 1999). Transgenic mice are particularly useful for both studying the consequences of disease-causing mutations in matrix genes and development of approaches to the gene therapy of the disorder. Since most mutations in OI are dominant negative, it is difficult to target them by gene therapy because supplying the normal gene without silencing the abnormal gene is ineffective. By elimination or reduction of the expression of mutant genes, gene therapy is able to create a functionally null allele and convert the clinically severe forms of OI into the biochemical equivalent of mild OI type I. One approach to mutation suppression involves the use of antisense oligonucleotides which bind to the target mRNA for the mutant protein and prevent its translation. Because of the highly repetitive nature of the type I collagen genes, the specificity of mutant allele suppression observed in the antisense oligonucleotide experiments appears to be insufficient for therapeutic trials (Wang & Marini, 1996). To increase specificity, an alternative approach of antisense therapy has been investigated using ribozymes. Selective cleveage of mutant type I collagen RNAs in cell-free assays (Grassi *et al.*, 1997) and in cultured OI fibroblasts (Dawson & Marini, 2000) was achieved using hammerhead ribozymes. Thus ribozymes seem to be promising future agents for the gene therapy of dominant negative genetic disorders, such as osteogenesis imperfecta.

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