Substitution of cysteine for glycine at residue 415 of one allele of the α1(I) chain of type I procollagen in type III/IV osteogenesis imperfecta

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Abstract
We have examined the type I collagen in a patient with type III/IV osteogenesis imperfecta. Two forms of α1(I) chain were produced, one normal and the other containing a cysteine residue within the triple helical domain of the molecule. Cysteine is not normally present in this domain of type I collagen. Peptide mapping experiments localised the mutation to peptide α1(I)CB3 which spans residues 403 to 551 of the triple helix. Subsequent PCR amplification of cDNA covering this region followed by sequencing showed a G to T single base change in the GGC codon for glycine 415 generating TGC, the codon for cysteine. The effect of the mutation on the protein is to delay secretion from the cell, reduce the thermal stability of the molecule by 2°C, and cause excessive post-translational modification of all chains in molecules containing one or more mutant α1(I) chains. The clinical phenotype observed in this patient and the position of the mutation conform to the recent prediction of Starman et al that Gly→Cys mutations in the α1(I) chain have a gradient of severity decreasing from the C-terminus to the N-terminus.

Osteogenesis imperfecta (OI) is an inherited connective tissue disease typified by excessive bone fragility. The major structural component of bone is type I collagen and molecular abnormalities of this protein have been identified in a number of OI patients. These abnormalities include major gene deletions,1,2 gene insertions,3 and mutations causing exon skipping,4 but most have been point mutations of glycine codons in either the α1(I) or α2(I) genes that disrupt the normal Gly-X-Y repeat sequence and destabilise the type I collagen molecule to varying degrees6-19 or occur in non-helical domains.20-22

Osteogenesis imperfecta is a heterogeneous syndrome which has to date been subclassified by clinical and radiological features as suggested by Silence et al.23 Only now, as more mutations are being identified, is it becoming feasible to correlate disease phenotype with the molecular abnormalities and their effect on the properties of the collagen molecule.

We report here the clinical phenotype and characterisation of the molecular defect of a patient with moderately severe osteogenesis imperfecta (OI III/IV) in whom one allele for the procI(I) chain of type I collagen harbours a glycine→cysteine mutation at position 415. (Numbering of residues follows the normal convention of beginning with the first residue of the triple helix.)

Materials and methods

CLINICAL SUMMARY
The patient was a male in his late fifties (fig 1). He was the only affected child of normal parents, with 10 sibs both younger and older than himself. He had a normal birth weight and length. His first recorded fracture occurred at 6 weeks of age. Over the next 16 years he suffered more than 270 fractures leading to progressive deformity of his skeleton. His sclerae were reported 'bluish' at birth but have become paler with age. (This is characteristic of type III OI.) He developed a conductive hearing loss in the third decade of life (not described previously in type III or type IV OI). Now edentulous, his teeth were reportedly yellowish-brown, suggesting dentinogenesis imperfecta. The skeletal radiographs (fig 2) showed generalised osteopenia with multiple fractures throughout the skeleton, including wedge

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compression fractures of the vertebral bodies. There was bowing and deformity of the long bones secondary to bone softening and a marked platybasia was noted.

**CELL CULTURE BLOST STUDIES**

Skin fibroblast cultures were established and maintained in Dulbecco's MEM medium (Flow Laboratories, Irvine, Scotland). Labelling of the cells with 14C-proline was normally carried out at 1 μCi ml⁻¹ as previously described. In experiments with short labelling times, the isotope concentration was increased to 5 μCi ml⁻¹ and for pulse chase experiments the initial pulse contained 2 μCi ml⁻¹ isotope. Before labelling with 35S-cystine, confluent cells were briefly incubated with cystine free Dulbecco's MEM and then labelled for 24 hours with the same medium containing 10 μCi ml⁻¹ 35S-cystine. Cells were incubated in a humidified atmosphere of 5% CO₂, 95% air at 37°C.

After labelling, medium and cells were harvested separately. The medium was mixed with proteinase inhibitors, N-ethylmaleimide (50 μg ml⁻¹), phenylmethyl sulphonyl fluoride (50 μg ml⁻¹), and EDTA (0-01 mol/l). Washed cells were trypsinised from the plate, pelleted, and lysed in either phosphate buffered saline containing 0.5 triton X100 (PBS-triton) or 0.5 mol/l acetic acid containing 0.5% triton X100 (acid-triton).

Proteins were precipitated from medium or PBS-triton cell extracts by addition of two volumes of absolute ethanol and incubation at 4°C overnight. Precipitates were collected by centrifugation at 6500 rpm for five minutes at 4°C in a microfuge. Pellets were washed three times with 66% aqueous ethanol, drained, and dried before redissolving in 0.5 mol/l acetic acid or SDS gel electrophoresis sample buffer.

Pepsin digestion of alcohol precipitates or acid-triton cell extracts was carried out in 0.5 mol/l acetic acid with 50 μg ml⁻¹ enzyme for six hours at 15°C. Digestion was stopped by addition of 0.5 μg ml⁻¹ pepstatin and lyophilisation.

Thermal stability measurements were carried out on 14C-proline labelled samples essentially as described.

Cyanogen bromide peptide mapping of α chains separated by gel electrophoresis was performed by the method of Barsh et al. using whole tracks or individual bands cut from the gel.

**GEL ELECTROPHORESIS**

Procollagen and collagen chains were separated on 5% polyacrylamide gels containing 2 mol/l urea using the buffer system of Laemmli. Cyanogen bromide peptides were separated on 10% acrylamide gels containing 0.5 mol/l urea. Radioactive peptides were visualised by autoradiography or fluorography using Fuji or Kodak x-ray film.

**ISOLATION OF CYTOPLASMIC RNA**

Cytoplasmic RNA was isolated from cultured fibroblasts (3 × 150 mm Petris) fed with medium containing fresh ascorbic acid 24 hours before harvesting. Extraction of RNA used the NP40 lysis technique.

**cDNA SYNTHESIS AND PCR AMPLIFICATION**

Primers used for PCR amplification were: 5′ sense primer 5′-CCTGGTCCTGATGGCAAAACT-3′ which corresponds to sequences within CB8 of the α(I) mRNA and 3′ antisense primer 5′-TTTGATCCCGAGACCAGCTGACCG3′ which is complementary to sequences within CB7 of the α(I) mRNA.

First strand cDNA synthesis used 5 μg total RNA in 25 μl of 1 × RT buffer (50 mmol/l Tris HCl, pH 8.3, at 42°C, 50 mmol/l KCl, 10 mmol/l MgCl₂, 1 mmol/l DTT, 10 μg ml⁻¹ BSA) containing 4 mmol/l sodium pyrophosphate, 20 units RNAsin, 1 mmol/l each dNTP, and 100 ng of 3′ antisense primer. Incubation with 20 units of AMV reverse transcriptase (Pharmacia) continued for 90 minutes at 42°C. One fifth of this reaction product was amplified for 40 cycles at 94°C for two minutes, 55°C for two minutes, and 65°C for two minutes using a 100 μl reaction containing 1 × PCR buffer (10 mmol/l Tris
Gly to Cys substitution in α1(1) in OI

HCl, pH 8.4, 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 400 μmol/l each dNTP, 100 ng each 5' and 3' primer, and 2.5 U Taq polymerase (Cetus). The reaction products were gel purified, blunt end ligated into M13mp18, and sequenced by the dideoxy chain termination method³ using sequenase (USB).

Results
Gel electrophoresis of pepsin derived medium collagens of cells from this patient (fig 3) showed an additional high molecular weight component migrating between α1(III) and α1(I). There was also evidence of slow migration of the α1(I) band in the patient compared to control collagens. If samples were reduced with mercaptoethanol before electrophoresis both the abnormal component and the α1(III) band disappeared without the appearance of any new bands (data not shown). Two dimensional electrophoresis of collagens run first unreduced then after reduction with mercaptoethanol (fig 4) showed that both α1(III) and the abnormal component migrated with the mobility of the α1(I) after reduction. This suggested that the high molecular weight component was a disulphide bonded dimer of α chains.

The nature of these α chains was investigated by in situ peptide mapping with cyanogen bromide (fig 5). The pattern for the dimer (*in fig 5A) clearly resembled that of α1(I) but there were some significant differences. Instead of a doublet for the α1CB7

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Figure 2. Skeletal radiographs of (A) skull, (B) spine, and (C) tibiae of patient.
and α1CB8, only a single broad spot was observed in the position of α1CB7. The α1CB3 spot was very faint and there was an additional spot above α1CB7 (arrowed in fig 5A, + MSH) not normally present in α1(I) maps (cf fig 5A and C, - MSH). Examination of the α1(I) region from the patient showed tilting of the bands for α1CB8, and the partial cleavage products α1CB5-8 and α1CB4-5-8 (seen very faintly above α1CB7) suggesting heterogeneity of these components owing to excessive post-translational modification. Other peptides appeared normal.

If after cyanogen bromide digestion the sample was reduced with mercaptoethanol before electrophoresis (fig 5B, + MSH) the spot arrowed in fig 5A disappeared and there was a decrease in the amount of partial cleavage products at the top of the gel but, unexpectedly, there was no major alteration to other peptides. These data suggested that the substitution was in α1CB3, but with α1CB3 not appearing after reduction the interpretation was not unequivocal. Cells were therefore labelled with 35S-cysteine, the procollagens precipitated, pepsin digested, and electrophoresed with and without previous reduction with mercaptoethanol (fig 6A).

Unlike 14C-proline labelling, where collagen is the predominant labelled species because of its high proline content, after labelling with 35S-cysteine the collagens were only a minor component of the total labelled protein even after pepsin digestion. When run unreduced (fig 6A, lane 1) there were several high molecular weight bands, but the α1(I) dimer (*) and α1(III)3 were clearly resolved. After reduction (fig 6A, lane 3) the mutant α1(I) and α1(III) ran together at the position of α1(I); all other labelled species migrated ahead of them in the gel. Bands corresponding to the 35S labelled α1 dimer (*), α1(III)3 (lane 1), and the α1(I) region of the reduced sample (lane 3), as well as 14C-proline labelled α1(III)3, α1(I), and α2(I) (lane 2), were excised and digested in situ with cyanogen bromide. In the maps obtained (fig 6B) the 35S labelled α1 dimer (lane c) showed a single band penetrating the gel (arrowed) migrating with the same relative mobility as the extra spot seen in the 14C-proline labelled maps (fig 5). There was also a significant amount of activity that did not penetrate the gel, which was assumed to be partial cleavage products containing disulphide bridges. In the reduced sample (lane d, fig 6B) several spots were seen and most of them were attributable to the type III collagen which contains two cysteine residues in its C-terminal peptide α1(III) CB9 and its partial cleavage products, for example, α1(III) CB5-9 (lanes d and e, fig 6B). In addition, there was a prominent spot in the position of α1(I)CB3 (large arrow, lane d) and a slightly less prominent spot corresponding to α1(I)CB8-3 (small arrow, lane d). This unequivocally localised the mutant cysteine to the CB peptide α1CB3 which...
contains residues 403 to 551 of the α1(I) chain (fig 6C).

To identify the precise mutation, cDNA for this region was amplified by PCR using flanking primers (fig 6C). A single fragment of 577 bp was obtained and subcloned into M13mp18. Several clones were picked and two distinct sequences were obtained in approximately equal abundance. One was normal but the other had the GGC codon for glycine 415 changed to TGC, the codon for cysteine (fig 7). After sequencing the entire fragment, only one other difference from the published sequence was observed. All clones had the sequence GGT for residue 517 instead of the published GGA. This change did not alter the protein sequence, being the third base of a glycine residue.

The effect of the mutation on certain physical and biological properties of the molecule was also investigated. The thermal stability of the molecule was reduced by approximately 2°C compared to controls (data not shown). Pulsing cells with isotope for periods up to four hours and analysing the medium and cells separately indicated that mutant molecules were poorly secreted and tended to accumulate within the cell (data not shown).

**Discussion**

Clinically the patient is difficult to classify according to the criteria of Sillence et al. He has a progressively deforming OI compatible with either OI type III or OI type IV phenotypes. With 10 normal sibs he is clearly a sporadic case, consistent with heterozygosity for a new dominant collagen mutation. Our analysis of the collagen produced by the patient's dermal fibroblasts identified the substitution of a cysteine residue in the triple helical domain of one α1(I) allele. The incorporation of two mutant chains in a type I collagen molecule (chain composition α1₂α2) leads to the formation of disulphide bonded...
$\alpha_1$ dimers. Peptide mapping experiments with $^{14}$C-proline labelled proteins, while confirming the mutant was an $\alpha_1$ chain, could not positively identify the site of the substitution. Two peptides $\alpha_1$CB8 and $\alpha_1$CB3 were missing from the dimer but, unexpectedly, neither reappeared on reduction of the disulphide bond. In retrospect this was probably owing to a combination of overmodification of $\alpha_1$CB8, causing it to migrate with $\alpha_1$CB7, and inefficient cleavage at the methionine residue between $\alpha_1$CB7 and $\alpha_1$CB3, possibly as a result of the proximity of the disulphide bond to the methionine (13 residues). However, specific labelling of the mutation with $^{35}$S-cysteine showed $\alpha_1$CB3 (residues 403 to 551 of the triple helical domain) contained the substitution. Analysis of the $\alpha_1$ mRNA for this region by PCR amplification of the cDNA showed heterozygosity for a single base change from a G to a T, altering the GGC codon for glycine 415 to TGC, the codon for cysteine. This substitution disrupts the Gly-X-Y-Gly-X-Y repeat sequence essential for the formation of a stable triple helical domain. As a result, type I collagen molecules containing mutant $\alpha$ chains have a reduced thermal stability (2°C below normal), have excessive post-translational modification amino-terminal of the substitution owing to delayed helix formation,29 and are poorly secreted from the cell. All these factors would combine to
produce an inadequate extracellular matrix and since type I collagen is the major structural component of bone would result in the osteopenia and bone fragility observed in this patient.

A number of Gly→Cys substitutions have now been identified in the α1 chain of type I collagen from OI patients, and the severity of the disease seems to depend on the position of the mutation within the molecule. Recently Starman et al\textsuperscript{12} have contrasted the phenotypic features of three such mutations. A Gly→Cys mutation at residue 718 (and others further C-terminal) resulted in lethal type II OI. Similar substitutions at residue 526 gave the OI III phenotype and at residue 94 produced the OI I phenotype. The results we have obtained with this patient are consistent with these observations and suggest that the varying clinical phenotype reflects subtle changes in the physical and biological properties of the mutant molecules.

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Figure 7  (A) DNA sequencing gel of cDNAs obtained by PCR amplification of α1CB3 of the patient’s α1(I) mRNA. (B) cDNA and amino acid sequence obtained for normal and mutant alleles.
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