Characterization of a type I collagen $\alpha 2(I)$ glycine-586 to valine substitution in osteogenesis imperfecta type IV

Detection of the mutation and prenatal diagnosis by a chemical cleavage method

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A chemical cleavage method for detecting mismatched bases in heteroduplexes formed between patient mRNA and control cDNA probes was employed to identify a single base mutation in a heterozygous case of osteogenesis imperfecta type IV. The parents' fibroblast mRNA did not contain the mutation. The region of the mRNA mismatch was amplified by using the polymerase chain reaction, cloned and sequenced. A point mutation of G to U at base-pair 2162 of the collagen $\alpha 2(I)$ mRNA resulted in the substitution of glycine by valine at amino acid position 586 of the helix. This substitution disrupted the critical Gly-Xaa-Yaa repeating unit of the collagen triple helix and resulted in helix destabilization, as evidenced by a decreased thermal stability. This local disturbance to helix propagation from the C-terminus to the N-terminus led to the overmodification of the collagen helix downstream towards the N-terminus. However, collagen secretion *in vitro* was normal, and the clinical phenotype probably resulted from the secretion into the extracellular matrix of the mutant collagen combined with a decrease in collagen production to 65 % of control values. The rapid detection of the osteogenesis imperfecta mutation by using the chemical cleavage method afforded the opportunity to apply the technique to prenatal diagnosis in the next pregnancy of the mother of the osteogenesis imperfecta patient. The absence of a mismatched base in chorionic villus mRNA and control cDNA heteroduplexes indicated that the foetus did not carry the mutation, which was confirmed by the subsequent delivery of a normal baby.

INTRODUCTION

Osteogenesis imperfecta (OI) is a heritable connective-tissue disorder in which bone fragility is the main clinical manifestation (Cole, 1988; Byers, 1989). There are four main clinical types, and mutations of type I collagen have been identified in each (Sillence *et al.*, 1979; Prockop & Kivirikko, 1984; Cheah, 1985; Byers, 1990). The mutations include point mutations, insertions, deletions and re-arrangements in the *COL1A1* gene that codes for the pro- $\alpha 1(I)$ gene that codes for the pro- $\alpha 1(I)$ chain and in the *COL1A2* gene that codes for the pro- $\alpha 1(I)$ chain of type I collagen (reviewed by Byers, 1990).

Many of the mutations result in the substitution of glycine residues within the large triple-helical domain of the α -chains. Glycine residues normally occupy each third position and are essential for the correct formation of the triple helix. Patients with glycine substitutions towards the C-terminus of the helix of the $\alpha 1(I)$ chain are likely to be more clinically severe than those with substitutions towards the N-terminus (Byers, 1990; Starman *et al.*, 1989). However, the type of α -chain involved, the nature and site of the substitution and the surrounding sequence may also contribute to the severity of the phenotype.

The proposal has been made that glycine substitutions in the $\alpha 2(I)$ chain lead to a milder phenotype than equivalent substitutions in the $\alpha 1(I)$ chain because of the stoichiometry of type I collagen molecules. In the usual heterozygous state found in OI, mutations of the $\alpha 2(I)$ chain would be expected to result in equal proportions of normal and mutant molecules, as type I collagen molecules contain only one $\alpha 2(I)$ chain. As type I collagen contains two $\alpha 1(I)$ chains, heterozygous mutations of this chain would be expected to result in only 25 % normal molecules, as 75 % of them would contain one or two mutant chains. The prediction of a milder phenotype with substitutions in the $\alpha 2(I)$ chain is supported by the substitution of Gly-1012 by arginine at

the junction of the helix and the C-telopeptide of the $\alpha 2(I)$ chain in a moderately severe form of OI, classified as OI-IV (Wenstrup *et al.*, 1988). However, the proposal is challenged by reports of helical substitutions of Gly-865 by serine and of Gly-907 by aspartic acid in lethal perinatal OI (Lamande *et al.*, 1989; Baldwin *et al.*, 1989). (Amino acid positions are numbered by the standard convention in which the first glycine residue of the triple-helical domain of the α -chain is number 1.)

In the present paper we report the substitution of Gly-586 by valine in the $\alpha 2(I)$ chain of type I collagen in a child with a moderately severe form of OI, classified as type IV-B.

EXPERIMENTAL

Clinical summary

The female child, OI50, had a progressively deforming type of OI with dentinogenesis imperfecta. As the parents were clinically normal and unrelated, she was classified as having OI type IV-B (Sillence *et al.*, 1979; Cole, 1988; Byers, 1989). Bone tissue was obtained from the patient and controls during routine orthopaedic procedures. Dermal fibroblast cultures were established from the parents and the patient, and a chorionic villus biopsy was undertaken for intra-uterine diagnosis at 11 weeks in the following pregnancy. All tissue biopsies were obtained with informed consent and approval of the Ethics Committee of this hospital. Fibroblast cultures established from the dermis and chorionic villus were maintained as described previously (Bateman *et al.*, 1984, 1986).

Extraction of bone collagens

Bone samples were freeze-milled and decalcified as previously described (Bateman *et al.*, 1986). Limited pepsin digestion was carried out, and the solubilized collagens were analysed by gel electrophoresis.

Abbreviation used: OI, osteogenesis imperfecta.

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Labelling of fibroblast collagens

Procollagens were biosynthetically labelled in confluent fibroblast cultures (Bateman *et al.*, 1984, 1986). After culture for 3 days in growth medium containing 0.25 mM-sodium ascorbate (Sigma), labelling with 10 μ Ci of L-[5- 3 H]proline (30 Ci/mmol; Amersham Corp.)/ml was carried out for 18 h in medium containing 10% (v/v) dialysed foetal-calf serum (Flow Laboratories), 0.25 mM-sodium ascorbate and 0.1 mM- β -aminopropionitrile fumarate (Sigma Chemical Co.). The cell layer and medium fractions were separated for analysis and the procollagens, precipitated with 25%-saturated $(\text{NH}_4)_2\text{SO}_4$, were subjected to limited proteolysis with pepsin (Bateman *et al.*, 1984, 1986).

SDS/PAGE

Collagen α -chains were resolved on 5% (w/v) separating gels containing 2 M-urea (Bateman *et al.*, 1984, 1986). Collagens prepared from cell cultures and tissues were also digested with CNBr and analysed by electrophoresis on 12.5% (w/v) gels (Scott & Veis, 1976; Bateman *et al.*, 1987b). The α -chains and peptides were detected by fluorography (Bonner & Laskey, 1974) or staining with Coomassie Brilliant Blue (Cole & Bean, 1979).

Thermal stability of collagen

The freeze-dried [3 H]proline-labelled collagens were dissolved in 0.4 M-NaCl/0.1 M-Tris/HCl buffer, pH 7.4, at 4 °C. The samples were warmed stepwise (1 °C/min) from 34 °C to 43 °C and, at 0.5 °C intervals, samples were taken and digested with a mixture of trypsin and chymotrypsin as described by Bruckner & Prockop (1981). The proportion of the collagen that was resistant to proteolysis at each temperature interval was determined by scintillation counting of the radioactivity of collagen α -chain bands that were resolved by electrophoresis and identified by fluorography (Bateman *et al.*, 1988b). The thermal denaturation temperature, T_m , was defined as the temperature at which half of the collagen was degraded.

Formation and chemical cleavage of mRNA·cDNA heteroduplexes

Total RNA was isolated from confluent fibroblast cultures (Lui *et al.*, 1979; Wake & Mercer, 1985). mRNA·cDNA heteroduplexes were formed in a volume of 50 μ l containing approx. 5 ng (50 000–100 000 d.p.m.) of labelled cDNA probe, 5–10 μ g of total RNA, 80% (v/v) formamide, 40 mM-Pipes buffer, pH 6.4, 1 mM-EDTA and 0.4 M-NaCl. The mixture was denatured at 80 °C for 5 min, incubated at 60 °C for 2 h and precipitated with ethanol. Chemical modification of mismatched nucleotides with hydroxylamine or osmium tetroxide, cleavage with piperidine and electrophoresis of the products on denaturing 7 M-urea/5% acrylamide gels have been described previously (Cotton *et al.*, 1988; Dahl *et al.*, 1989; Lamande *et al.*, 1989; Bateman *et al.*, 1989).

The apparent site of the mutation in the collagen chains was used as a guide to the selection of suitable control cDNAs for heteroduplex formation. Control $\alpha 1(\text{I})$ and $\alpha 2(\text{I})$ cDNA probes, chosen to span the abnormal region, were purified and end-labelled with [α - 32 P]dCTP by the fill-in reaction using the Klenow fragment of DNA polymerase I (Sambrook *et al.*, 1989).

Amplification and sequencing of cDNA

First-strand cDNA was synthesized from total RNA by using a cDNA synthesis kit (Amersham Corp.) primed with 50 ng of a specific $\alpha 2(\text{I})$ oligonucleotide, 5'-GACCAGTTTCACACGGTTT-3', which was 3' to the mismatched nucleotide. The primer corresponded to nucleotide residues 3063–3082 of normal $\alpha 2(\text{I})$ cDNA (de Wet *et al.*, 1987).

[The base-pairs are numbered from the start of transcription of the pro- $\alpha 2(\text{I})$ mRNA (de Wet *et al.*, 1987).] Approx. 50 ng of cDNA was amplified by the PCR (Saiki *et al.*, 1988) through 30 cycles with the use of *Taq* polymerase (Perkin-Elmer-Cetus) (Kogan *et al.*, 1987). Each cycle consisted of denaturation at 92 °C for 1.5 min, annealing of primers at 62 °C for 1.5 min and primer extension at 72 °C for 3 min (Lamande *et al.*, 1989; Bateman *et al.*, 1989). A 371 bp fragment corresponding to bases 1984–2355 of the $\alpha 2(\text{I})$ cDNA was amplified with the primers 5'-CCTGATGGAAACAAGGGTGA-3' and 5'-TTCTCCTTTAGCACCCGGTT-3' (de Wet *et al.*, 1987).

Amplification products of the predicted size were purified, treated with T4 polynucleotide kinase (Sambrook *et al.*, 1989) and cloned into a *Sma*I-cut dephosphorylated M13mp8 vector (Amersham Corp.). Multiple clones from two independent amplification reactions were sequenced by using a Sequenase kit (United States Biochemical Corp.).

RESULTS

Protein abnormalities

Abnormally slow electrophoretic mobilities of the $\alpha 1(\text{I})$ and $\alpha 2(\text{I})$ chains were observed in type I collagen extracted from OI50 bone (results not shown). This migrational abnormality was also evident in labelled collagen from the cell layer and medium of cultured OI50 fibroblasts (Fig. 1, lane 2). In contrast, the collagen α -chains produced by fibroblasts from both parents migrated normally (Fig. 1, lanes 3 and 4). Type I collagen secretion by OI50 fibroblasts was apparently normal (Bateman *et al.*, 1988a).

The slow migration of the OI50 α -chains was probably due to overmodification of lysine residues on the N-terminal side of the mutation (Bateman *et al.*, 1984, 1986). CNBr-cleavage peptide

Fig. 1. Electrophoresis of pepsin-digested fibroblast collagens

Fibroblast cultures were labelled for 18 h with [3 H]proline, and the collagens secreted into the medium were pepsin-digested and analysed without reduction by electrophoresis on SDS/polyacrylamide gels (5%) (see the Experimental section for details). Lane 1, control; lane 2, OI patient; lane 3, father; lane 4, mother. The migration positions of type I collagen $\alpha 1(\text{I})$ and $\alpha 2(\text{I})$ chains and type III collagen [$\alpha 1(\text{III})$] $_3$ are shown.

Fig. 2. Electrophoresis of the CNBr-cleavage products of fibroblast collagen

OI50 and control fibroblasts were labelled in cell culture, and collagens in the medium fraction were digested with CNBr and the products analysed on 12.5% (w/v) polyacrylamide gels (see the Experimental section for details). Lane 1, control; lane 2, OI50. The major $\alpha 1(I)$ and $\alpha 2(I)$ CNBr-cleavage peptides are indicated.

maps showed slow migration of the $\alpha 2(I)$ CB3.5 peptide (Fig. 2), suggesting that the mutation causing helix perturbation and consequent overmodification of lysine residues was in the central region of either the $\alpha 2(I)$ or $\alpha 1(I)$ chain.

There were no apparent abnormalities in type III or V collagens (results not shown).

Detection of an abnormal mRNA sequence by chemical cleavage

Heteroduplexes were formed with control $\alpha 2(I)$ and $\alpha 1(I)$ cDNAs, which spanned the central portion of the collagen helix, and mRNA extracted from cultured dermal fibroblasts of OI50 and controls. Hydroxylamine/piperidine treatment cleaved a 300 bp fragment from the 910 bp *NcoI*-*XhoI* fragment of $\alpha 2(I)$ cDNA. This finding suggested that there was a mismatched C at approximately base-pair 2161 of the control cDNA (Fig. 3). Treatment with osmium tetroxide/piperidine did not reveal any T mismatches (Fig. 3). No mismatches were detected by hydroxylamine or osmium tetroxide treatment of heteroduplexes formed between OI50 mRNA and control $\alpha 1(I)$ cDNA (results not shown).

Characterization of the mRNA mutation

The chemical cleavage method of detecting sequence mismatches defined the position and the mismatched base in the control $\alpha 2(I)$ cDNA probe but did not define the precise base change in the mutant mRNA. To define the mutation, a short length of first-strand $\alpha 2(I)$ cDNA was amplified by the PCR with unique oligonucleotides chosen to span the region containing the mismatch.

Sequencing of M13 subclones of the amplified products identified the mutation as a single base substitution that converted the codon for glycine (GGT), at position 586 of the helix, into GTT (valine) (Fig. 4). The abnormal sequence was confirmed by sequencing of multiple clones from two separate PCRs. Clones

Fig. 3. Detection of the $\alpha 2(I)$ mutation in the OI mRNA by chemical modification and cleavage

Total RNA from control and OI50 fibroblasts was hybridized to a 910 bp *NcoI*-*XhoI* ^{32}P -labelled fragment of the $\alpha 2(I)$ cDNA and allowed to react with hydroxylamine or osmium tetroxide for 0–60 min as indicated, then treated with piperidine, and the resultant fragments were resolved on a 5% (w/v) polyacrylamide gel (see the Experimental section for details). The labelled $\alpha 2(I)$ cDNA fragment produced by cleavage at reactive Cs by hydroxylamine (300 bp) and the uncleaved probe (910 bp) are indicated. The molecular-mass standards used were ^{32}P -labelled *HaeIII* fragments of ϕX174 DNA.

containing the normal sequence (GGT) were also identified, indicating that the patient was heterozygous for the $\alpha 2(I)$ mutation.

The codon for amino acid 588 of the triple-helical domain of the $\alpha 2(I)$ chain was CCT (proline) in the normal and mutant amplified cDNAs of OI50 as well as in control cDNAs, rather than the published codon, CAT (histidine) (de Wet *et al.*, 1987).

Melting temperature of collagen

The thermal denaturation temperature (T_m) of type I collagen synthesized by OI50 and control fibroblasts was estimated by measuring its resistance to proteinase digestion after heating to different temperatures (Fig. 5). The T_m of OI50 type I collagen was approx. 40.5 °C compared with 41.3 °C for control type I collagen. In contrast, the T_m of type III collagen from OI50 was normal (results not shown).

Prenatal diagnosis

The collagens produced by cultured chorionic villus cells were compared with the collagens produced by control chorionic villus cells and dermal fibroblasts. The cells grown from the chorionic villus produced type I collagen $\alpha 1(I)$ and $\alpha 2(I)$ chains, but the $\alpha 1/\alpha 2$ ratio was much higher than that of control fibroblasts (results not shown). This indicated that the type I

Fig. 4. DNA sequence of mutant and normal cDNA clones

cDNA clones of the PCR-amplified mRNA region containing the mismatched base were sequenced (see the Experimental section for details). The OI50 mutant clone contains a G to T substitution at base position 2160 converting Gly-586 into valine. In both mutant and normal clones base position 2168 was C, so that amino acid 588 was proline rather than histidine (de Wet *et al.*, 1987). Nucleotides different from the published sequence are boxed.

collagen population contained a significant proportion of $\alpha 1(I)$ homotrimeric molecules as well as heterotrimeric type I collagen molecules with the normal chain composition, $[\alpha 1(I)]_2\alpha 2(I)$. Since

normal $\alpha 1(I)$ trimer molecules have increased levels of post-translational modifications (Uitto, 1979), prenatal diagnosis by detection of slow migration of the α -chains due to underlying structural defects was not possible.

However, since the cells produced $\alpha 2(I)$ mRNA, the chemical cleavage method of detecting point mutations could be used (Dahl *et al.*, 1989). RNA was extracted from cells cultured from chorionic villus, OI50 dermis and the parents' dermis. Chemical cleavage of $\alpha 2(I)$ mRNA·cDNA heteroduplexes again showed an abnormal cleavage product in the patient (OI50) but not in the foetal or parental samples (Fig. 6). We concluded that the foetus did not have the OI50 mutation and this proposal was confirmed by the delivery of a normal full-term baby.

DISCUSSION

A heterozygous point mutation of G to T was identified at position 2162 of the $\alpha 2(I)$ cDNA in this case of OI type IV-B. The deduced amino acid change consisted of the substitution of Gly-586 by valine in the $\alpha 2(I)$ chain of type I collagen. It probably represented a new sporadic mutation in the patient as neither parent had clinical evidence of OI or evidence of the mutation in dermal fibroblasts. Mosaicism in one parent, which has been described in OI (Byers *et al.*, 1988; Wallis *et al.*, 1990), was not excluded.

Our previous studies showed that type I collagen production was reduced to about 65% of control values, although the proportion secreted was normal (Bateman *et al.*, 1988a). Electrophoretic analysis of the α -chains and CNBr-cleavage peptides was not able to detect directly the substitution of Gly-586 by valine, but indirect evidence of the mutation was found. The evidence consisted of the slow migration of all the α -chains and selected CNBr-cleavage peptides of type I collagen. The slow migration of the α -chains was likely to be due to enzymic overmodification of lysine residues on the N-terminal side of the glycine substitution (Bateman *et al.*, 1984; Bonadio & Byers, 1985). As the child was heterozygous for the mutation, we

Fig. 5. Thermal denaturation of type I collagen

[^3H]Proline-labelled collagens were prepared from the medium of OI50 and control fibroblasts. They were treated with pepsin, and after inactivation of the pepsin the collagens were warmed slowly from 34 °C to 43 °C. Serial samples were taken and digested with trypsin/chymotrypsin, and the resistant chains were resolved by electrophoresis: (a) upper panel, control; lower panel, OI50. The migration positions of $\alpha 1(I)$ and $\alpha 2(I)$ chains are shown. The 'melting' temperature of the helix was determined by the relative proportion of the α -chains that were resistant to proteolysis (see the Experimental section for details). (b) $\square$, Type I collagen from OI50 [$\alpha 2(I)$ Gly-586 to valine]; $\circ$, type I collagen from control fibroblasts.

Fig. 6. Prenatal diagnosis using the chemical cleavage method

Total RNA from OI50 fibroblasts, parental fibroblasts and chorionic villus fibroblasts from a subsequent pregnancy were hybridized to a 910 bp *NcoI-XhoI* ^{32}P -labelled fragment of the $\alpha 2(I)$ cDNA and allowed to react with hydroxylamine or osmium tetroxide, treated with piperidine and the resultant fragments resolved by electrophoresis on a 5% (w/v) polyacrylamide gel under conditions identical with those used in Fig. 3 (see the Experimental section for details). The labelled $\alpha 2(I)$ cDNA fragment was cleaved at a reactive C by hydroxylamine (300 bp) in OI50 (see Fig. 3) whereas the heteroduplex formed with parental or foetal mRNA remained uncleaved, indicating that the OI50 mutation was not carried by the parents or by the foetus. The molecular-mass standards used were ^{32}P -labelled *HaeIII* fragments of ϕX174 DNA.

expected that about half the molecules would contain a mutant $\alpha 2(I)$ chain and be overmodified while the remaining molecules would be normal. The explanation for the discrepancy between the observed and expected results was not determined. However, similar discrepancies have also been observed in some of the OI patients with glycine substitutions in the $\alpha 1(I)$ chain, where only slowly migrating collagen was identified (Bateman *et al.*, 1984, 1986), although the patients were heterozygous and expressed normal as well as mutant alleles (Bateman *et al.*, 1987a; Lamande *et al.*, 1989).

The functional consequences of glycine substitutions in any of the repeating Gly-Xaa-Yaa triplets of the helical domain of the α -chains may depend on the type of α -chain involved, the nature and site of the substitution and the surrounding sequence. The glycine substitution in OI50 had a relatively small helix-destabilizing effect. In OI50, the thermal stability was reduced by 0.8 $^{\circ}\text{C}$, whereas reductions of 1–1.5 $^{\circ}\text{C}$ were usual with glycine substitutions in the $\alpha 1(I)$ chain (Baker *et al.*, 1989; J. F. Bateman, M. Hannagan, D. Chan & W. G. Cole, unpublished work). This smaller reduction in the T_m probably reflects the $[\alpha 1(I)]_2\alpha 2(I)$ stoichiometry of the helix, where $\alpha 2$ mutations could be expected to be, in general, less disruptive than those in $\alpha 1$.

The sequence surrounding the glycine substitution may have

also contributed to the small helix-destabilizing effect. The local sequence around $\alpha 2(I)$ Gly-586 contains only one potentially hydroxylatable proline residue at position 588. The corresponding region of the $\alpha 1(I)$ chain is also largely devoid of hydroxylatable proline residues and thus this region of the type I collagen helix may be relatively 'loose', as hydroxyproline residues stabilize the helix (Burjanadze, 1979). This 'looseness' of the normal helix may mean that the substitution of Gly-586 has a less disruptive effect than if it occurred in a different region of the $\alpha 2(I)$ chain.

Three babies with mutations resulting in the substitution of glycine by valine in the helical domain of $\alpha 1(I)$ chains have been reported (Lamande *et al.*, 1989; Patterson *et al.*, 1989). They were lethal perinatal cases of OI (OI-II) with substitution of Gly-1006, Gly-973 or Gly-256. The baby with the more N-terminal substitution survived longer than the other babies, which may indicate that a gradient of clinical severity exists from the C-terminal substitution to more N-terminal one. A gradient of clinical severity, from lethal OI-II cases to mild OI-I cases, has also been reported for substitutions of glycine by cysteine in the helix of the $\alpha 1(I)$ chain (Starman *et al.*, 1989).

Too few glycine substitutions have been reported in the $\alpha 2(I)$ chain to be able to make any useful correlations between the site and type of $\alpha 2(I)$ substitutions and their phenotypic consequences. However, the identification of glycine substitutions in patients with type II and type IV OI suggests that a similar range of phenotypes to those found with $\alpha 1(I)$ substitutions will be found. Further studies should help to reveal functionally important domains within the type I collagen $\alpha 1(I)$ and $\alpha 2(I)$ chains.

Intra-uterine diagnosis was successfully undertaken in the subsequent pregnancy. When we undertook these studies, we had localized the sequence abnormality in $\alpha 2(I)$ mRNA, using the chemical cleavage method of detecting sequence mismatches in mRNA-cDNA heteroduplexes, but we had not sequenced the mutation. As a result, the chemical cleavage method was used to screen directly for the mutation in foetal $\alpha 2(I)$ mRNA obtained from cultured chorionic villus cells. The absence of an abnormal cleavage product from the foetal sample indicated that the foetus did not carry the mutation. The use of the chemical cleavage method for prenatal diagnosis was much more definitive than protein analysis on chorionic villus cells. Protein analyses rely on the indirect assessment of the mutation by abnormal electrophoretic migration, and, in this case, the presence of slowly migrating $\alpha 1$ trimer molecules made interpretation of the result ambiguous. Thus the chemical cleavage method represents a convenient diagnostic strategy when a mismatch has been identified in the previously affected individual. In many cases this may also prove to be a preferable strategy to the alternative molecular approaches of PCR amplification of the region containing the mutation followed by sequencing or allele-specific restriction mapping.

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