Biochem. J. (1984) **217**, 103–115
Printed in Great Britain

Abnormal type I collagen metabolism by cultured fibroblasts in lethal perinatal osteogenesis imperfecta

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(*Received 7 June 1983/Accepted 31 August 1983*)

Cultured skin fibroblasts from seven consecutive cases of lethal perinatal osteogenesis imperfecta (OI) expressed defects of type I collagen metabolism. The secretion of [^{14}C]proline-labelled collagen by the OI cells was specifically reduced (51–79% of control), and collagen degradation was increased to twice that of control cells in five cases and increased by approx. 30% in the other two cases. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed that four of the OI cell lines produced two forms of type I collagen consisting of both normally and slowly migrating forms of the α1(I)- and α2(I)-chains. In the other three OI cell lines only the 'slow' α(I)'- and α2(I)'-chains were detected. In both groups inhibition of the post-translational modifications of proline and lysine resulted in the production of a single species of type I collagen with normal electrophoretic migration. Proline hydroxylation was normal, but the hydroxylysine contents of α1(I)'- and α2(I)'-chains purified by h.p.l.c. were greater than in control α-chains. The glucosylgalactosylhydroxylysine content was increased approx. 3-fold while the galactosylhydroxylysine content was only slightly increased in the α1(I)'-chains relative to control α1(I)-chains. Peptide mapping of the CNBr-cleavage peptides provided evidence that the increased post-translational modifications were distributed throughout the α1(I)'- and α2(I)'-chains. It is postulated that the greater modification of these chains was due to structural defects of the α-chains leading to delayed helix formation. The abnormal charge heterogeneity observed in the α1 CB8 peptide of one patient may reflect such a structural defect in the type I collagen molecule.

Osteogenesis imperfecta (OI) is a heterogeneous group of genetically determined disorders of the connective tissues in which bone fragility is the main clinical feature. Patients with OI have been classified, according to their clinical features and inheritance patterns, into four groups by Sillence *et al.* (1979). It is apparent, however, that heterogeneity exists within these groups and it is probable that such diversity also exists in the underlying molecular defects. It is likely that defects of type I collagen form the molecular basis of OI (see reviews by Eyre, 1981; Byers *et al.*, 1982), although direct evidence of such defects has only been reported in a few patients (Nicholls *et al.*, 1979; Peltonen *et al.*, 1980; Barsh & Byers, 1981; Barsh *et al.*, 1982; Byers *et al.*, 1983).

Abbreviations used: OI, osteogenesis imperfecta; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate; h.p.l.c., high-performance liquid chromatography.

With a view to defining some of the molecular defects of type I collagen in OI we have studied the metabolism of collagen in the lethal, perinatal form of this disease (Sillence *et al.*, 1979). We show that cultured skin fibroblasts obtained from seven patients secreted reduced amounts of collagen, degraded increased amounts of collagen and synthesized abnormal type I collagen. We also show that heterogeneity exists in each of these parameters.

Experimental

Materials

L-[4-^{3}H]Proline (15.7 Ci/mmol), L-4-[G-^{3}H]-hydroxyproline (5 Ci/mmol) and L-[U-^{14}C]proline (293 mCi/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A., and L-[5-^{3}H]-proline (27 Ci/mmol), L-[U-^{14}C]lysine (340 mCi/mmol) and PCS liquid scintillation solution were purchased from Amersham Australia Pty., Syd-

ney, NSW, Australia. Dulbecco's modification of Eagle's medium (Dulbecco & Freeman, 1959), and foetal calf serum were obtained from Flow Laboratories Australasia, Stanmore, NSW, Australia. Chromatographically purified bacterial collagenase was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. and further purified (Peterkofsky & Diegelmann, 1971). Pepsin, β-aminopropionitrile fumarate and sodium ascorbate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. *N*-Ethylmaleimide, α,α′-dipyridyl and trifluoroacetic acid were purchased from BDH Chemicals, Poole, Dorset, U.K., and phenylmethanesulphonyl fluoride was obtained from Merck, Darmstadt, West Germany. Pepstatin was obtained from Boehringer, Mannheim, West Germany, acetonitrile was purchased from Waters Associates, Milford, MA, U.S.A., and Dowex AG 50W X8 cation exchange resin (200–400 mesh) was obtained from BioRad Laboratories, Richmond, CA, U.S.A. Galactosylhydroxylysine and glucosylgalactosylhydroxylysine standards were generously provided by Dr. S. Krane, Harvard Medical School, Boston, MA, U.S.A. All other chemicals were commercially available analytical grade reagents.

Dermal fibroblast cultures

Dermal fibroblast cultures were established from skin biopsies obtained at autopsy from seven consecutive cases of lethal perinatal osteogenesis imperfecta and from the parents of four of these cases. The babies showed the typical features of this type of OI. The long bones were broad and crumpled but in one baby (OI 26) the long bones had a more normal shape. Skin biopsies were also obtained from age-matched control subjects who had died from diseases that did not affect the connective tissues. The biopsies were obtained with the approval of the Ethics Committee of this Hospital and with informed parental consent.

Fibroblast cultures between the third and seventh passages were grown in Dulbecco's modification of Eagle's medium containing 20mM-Hepes/22mM-$NaHCO_3$, pH7.60, 2mM-L-glutamine and 10% (v/v) foetal calf serum, in an atmosphere of air/CO_2 (19:1) at 37°C. The medium was changed every 3 days and when the cells had reached confluency the cultures were trypsinized and the cells were transferred to Petri dishes for experimental determinations. Antibiotics were not added to the growth media and all cultures were free of mycoplasma contamination.

Collagen production, secretion and proline hydroxylation

The incorporation of [^{14}C]proline into collagenase-digestible protein was used to quantify collagen production and secretion (Peterkofsky & Diegelmann, 1971) and proline hydroxylation was measured simultaneously by the inclusion of [4-^{3}H]proline in the labelling medium (Chojkier *et al.*, 1980). Loss of ^{3}H from L-[4-^{3}H]proline due to hydroxylation at the 4-position causes a reduction in the $^3H/^{14}C$ ratio of collagen, the magnitude of this reduction reflecting the proportion of proline residues hydroxylated.

Fibroblasts were seeded into four 28cm^2 plastic Petri dishes at 2×10^5 cells/dish and incubated in 10ml of growth medium until the cells had been confluent for 1–2 days. The medium was removed and replaced with 4.9ml of Dulbecco's modification of Eagle's medium, buffered with Hepes/$NaHCO_3$, pH7.60, as above, containing 2mM-glutamine, 0.15mM-sodium ascorbate and 10% (v/v) dialysed foetal calf serum. Preincubation was performed at 37°C in an atmosphere of air/CO_2 (19:1). After 4h, 0.1ml of a mixture of 2μCi of L-[U-^{14}C]proline and 10μCi of L-[4-^{3}H]proline containing unlabelled proline was added to the medium and the incubation was continued for a further 18h. The final concentration of proline in the medium was 0.1mM. The proline isotope mixture had been purified before use by Dowex AG 50W ion-exchange chromatography to remove ^3H_2O and trace amounts of other radioactively labelled contaminants (Berg *et al.*, 1980). With these culture conditions the incorporation of [^{14}C]-proline into collagen by control cells was linear for 24h and collagen secretion and proline hydroxylation were normal throughout the incubation.

Following incubation, the cell layer and medium fractions were treated separately. The culture dishes were rapidly chilled on ice and the medium from each of the four dishes was collected and combined with a 2ml phosphate-buffered saline (0.1M-NaH_2PO_4/0.15M-NaCl, pH7.4) rinse of the cell layers. All subsequent procedures, unless otherwise stated, were performed at 0–4°C. The precipitation of collagenous and non-collagenous proteins in the medium with 10% (w/v) trichloroacetic acid, as used in the Peterkofsky & Diegelmann (1971) collagenase assay, resulted in the co-precipitation of large quantities of foetal calf serum proteins. As the precipitated serum proteins interfered with the collagenase assay, an initial selective precipitation of collagenous proteins was used. To the pooled medium fraction, 0.5ml of a 2mg/ml solution of human type I collagen was added followed by $(NH_4)_2SO_4$ to a final concn. of 25% saturation. After 16h the collagens had precipitated along with high-molecular-weight non-collagenous proteins. The precipitate was collected by centrifugation for 30min at 40000*g* and washed three times with 1ml of 5% (w/v) trichloroacetic acid, containing 1mM-proline. The precipitate was dissolved in

0.6ml of 0.2M-NaOH and the incorporation of radioactivity into collagenous and high-molecular-weight non-collagenous protein was assayed in 0.2ml portions by the specific collagenase assay of Peterkofsky & Diegelmann (1971) as modified by Chojkier *et al.* (1980).

The low-molecular-weight non-collagenous proteins synthesized by the cells were soluble in $(NH_4)_2SO_4$ at 25% saturation. These proteins were precipitated with 10% (w/v) trichloroacetic acid and the precipitate was collected by centrifugation (10000*g* for 5min). The precipitate was washed four times with 10% (w/v) trichloroacetic acid/1mM-proline and was hydrolysed in 1ml of 6M-HCl at 110°C for 20h. Fractions (0.1ml) were assayed for radioactivity to determine the incorporation of [^{14}C]proline into low-molecular-weight non-collagenous proteins. The serum proteins in the precipitate did not interfere with this analysis. The total incorporation of [^{14}C]proline into the non-collagenous proteins of the medium was calculated by adding the incorporations of the radioactivity into the high- and low-molecular-weight fractions.

The cell layer was scraped from each culture dish into 3ml of 0.05M-Tris/HCl, pH7.5, containing 0.15M-NaCl. The dishes were rinsed with a further 3ml of this Tris/HCl solution and the combined cell layer fraction was disrupted by sonication with an MSE instrument equipped with a 3mm diameter needle probe (3 × 15s, 10μm peak-to-peak). The proteins were precipitated with 10% (w/v) trichloroacetic acid and washed three times with 1ml of 5% (w/v) trichloroacetic acid/1mM-proline. The precipitates were dissolved in 0.6ml of 0.2M-NaOH and 0.2ml fractions were assayed for collagenous and non-collagenous proteins (Peterkofsky & Diegelmann, 1971; Chojkier *et al.*, 1980).

The incorporation of [^{14}C]proline into collagenous and non-collagenous proteins was corrected for differing cell densities by measuring the DNA content (Burton, 1956) of portions of the 0.2M-NaOH solution of the cell layer.

Collagen degradation

The degradation of collagen was determined by measuring the amount of [^{14}C]hydroxyproline appearing free or in low-molecular-weight peptides relative to the amount in collagen molecules after labelling with [^{14}C]proline, using a simplified form of the method described by Bienkowski & Engels (1981).

Fibroblasts were grown and incubated in duplicate with 2μCi of L-[U-^{14}C]proline per dish, as described above. The labelled proline was purified before use to remove traces of contaminating [^{14}C]-hydroxyproline (Berg *et al.*, 1980). After an 18h incubation the cell and medium fractions were combined for analysis. The proteins were precipitated with 75% (v/v) ethanol and after 2h the supernatant and precipitate were collected by centrifugation (1000*g* for 5min). After addition of [^{3}H]hydroxyproline as a recovery standard, the supernatants were hydrolysed under N_2 in 6M-HCl at 110°C for 20h and then filtered and dried. The samples were dissolved in 2ml of water, applied to a column (1cm × 20cm) containing Dowex AG 50W X8 (H^+ form) resin and eluted with 1M-HCl. The fractions containing hydroxyproline were collected and dried. This preliminary step was included to remove most of the excess [^{14}C]proline present in this fraction. The samples were dissolved in 0.1M-HCl and loaded on to a column (0.32cm × 50cm) packed with Technicon C3 sulphonated polystyrene. The column was equilibrated at 45°C with sodium citrate buffer, pH2.67, containing 0.2M-Na^+, 6% (v/v) methanol and 0.05% (w/v) disodium EDTA and was eluted at a flow rate of 0.5ml/min. Fractions (0.5ml) were collected into vials and assayed for radioactivity using dual-label counting techniques. Radioactivity was only detected in the hydroxyproline and proline peaks which were completely resolved from aspartate, glutamate and other proline metabolites (Chojkier *et al.*, 1982). [^{14}C]Hydroxyproline in the hydrolysed supernatant was a measure of the amount of degraded collagen.

The proteins precipitated with 75% (v/v) ethanol were redissolved in 0.05M-Tris/HCl, pH7.5, containing 0.15M-NaCl and macromolecular collagen was precipitated by addition of $(NH_4)_2SO_4$ to a final concentration of 25% saturation. After 16h at 4°C the precipitate was collected by centrifugation (40000*g* for 30min), and following addition of [^{3}H]hydroxyproline as a recovery standard, hydrolysed under N_2 in 6M-HCl at 110°C for 20h, filtered and dried. The samples were redissolved in 0.1M-HCl and [^{14}C]hydroxyproline was quantified on the amino acid analyser as described above. [^{14}C]Hydroxyproline in this fraction was a measure of the amount of macromolecular collagen.

Preparation of labelled procollagen and collagen

The labelling conditions described above were used except that 50μCi of L-[5-^{3}H]proline was added to each dish and 0.1mM-β-aminopropionitrile fumarate was included in the medium to inhibit collagen cross-linking. In the experiments designed to determine lysine hydroxylation and glycosylation, the [^{3}H]proline was replaced with 10μCi of L-[^{14}C]lysine. Also, in some experiments α,α'-dipyridyl was added to the culture medium, at a final concentration of 0.1mM, to prevent lysine and proline hydroxylation.

The medium and cell layers were prepared, as

already described for our studies of collagen production, with the inclusion of 0.1mM-phenylmethanesulphonyl fluoride, 10mM-*N*-ethylmaleimide and 25mM-EDTA to prevent proteolytic degradation of procollagen. The procollagens were precipitated by $(NH_4)_2SO_4$, as described previously, after the addition of 50μg of human type I collagen as carrier. The precipitate was collected and redissolved in 2ml of 0.05M-Tris/HCl, pH7.5, containing 0.15M-NaCl and 1mM-EDTA. Portions of the procollagen solutions were mixed with an equal volume of 1M-acetic acid and limited pepsin digestion was performed at a final pepsin concentration of 50μg/ml for 6h at 4°C. Digestion was terminated by the addition of a 100-fold molar excess of pepstatin and the collagens were precipitated by the addition of an equal volume of 0.5M-acetic acid containing 4M-NaCl. After stirring at 4°C for 16h, the solution was centrifuged (40000*g* for 30min) and the precipitated collagen was washed with 75% (v/v) ethanol and freeze-dried. In other portions of the procollagen solutions that were to be used for peptide map analysis, the procollagens were precipitated from the solution by the addition of ethanol to 75% (v/v) and the precipitate was collected by centrifugation (2000*g* for 5min) and freeze-dried.

Purification of α-chains by reverse-phase h.p.l.c.

The freeze-dried pepsin-digested procollagens, with 50μg of human type I collagen as carrier, were dissolved in 0.15ml of 0.1% (v/v) trifluoroacetic acid containing 12.5% (v/v) acetonitrile. The solution was filtered and the collagen was denatured at 60°C for 10min. Chromatography was performed using a 30nm pore, C_{18} reverse-phase Vydac TP201 column (4.6mm × 250mm), obtained from the Separations Group, Hesperia, CA, U.S.A. Collagen chains were separated at room temperature in aqueous 0.1% (v/v) trifluoroacetic acid with a linear gradient of 12.5% (v/v) to 37.5% (v/v) acetonitrile over 90min. The eluent flow rate was 1.2ml/min; 0.5min fractions were collected. Using these chromatographic conditions, which were similar to those employed by van der Rest & Fietzek (1982), the α1(I)-, α2(I)- and $[\alpha1(III)]_3$-chains were resolved completely.

Determination of lysine hydroxylation and glycosylation

The [^{14}C]lysine-labelled α1(I)- and α2(I)-chains that were resolved by h.p.l.c. were pooled and freeze-dried. Lysine hydroxylation was determined on an amino acid analyser after hydrolysis in 6M-HCl under N_2 at 110°C for 20h. Alkaline hydrolysis with 2M-KOH was also performed to determine the proportion of glycosylated hydroxylysine residues (Pinnell *et al.*, 1971).

SDS/polyacrylamide-gel electrophoresis

Electrophoretic analysis of procollagen and collagen chains were performed on 5% (w/v) separating gels with a 3.5% (w/v) stacking gel. The sample preparation and electrophoresis conditions are described elsewhere (Laemmli, 1970; Bateman & Peterkofsky, 1981). The radioactivity in the protein bands was determined by fluorography (Bonner & Laskey, 1974) and quantified by densitometric scanning of the fluorograms (Quick-Scan, Helena Laboratories, Beaumont, TX, U.S.A.). X-ray film (Kodak XAR-5) was 'pre-flashed' to obtain a linear relationship between the radioactivity and image intensity of the bands (Laskey & Mills, 1975).

Analysis of CNBr-cleavage peptides

Freeze-dried samples of procollagen, collagen and α-chains were dissolved in 70% (v/v) formic acid containing 50mg of CNBr/ml and cleavage was achieved by the method of Scott & Veis (1976). The released peptides were resolved by one-dimensional and two-dimensional polyacrylamide-gel electrophoresis (Cole & Bean, 1979; Cole & Chan, 1981). The two-dimensional technique combines non-equilibrium pH-gradient-gel electrophoresis in the first dimension and SDS/polyacrylamide-gel electrophoresis in the second dimension, thus separating the peptides on the basis of their charge and molecular weight.

Results

Proline hydroxylation and collagen degradation

The hydroxylation of proline in the collagens synthesized by the OI cells was normal. The intracellular collagens from the OI cells had 47.4 ± 1.6% of their proline residues hydroxylated compared with 48.3 ± 2.3% in the control cells. The extracellular collagens from the OI cells had values of 47.3 ± 2.3% compared with 46.5 ± 3.1% in the control cells. These results are the mean ± S.D. of fourteen separate determinations.

In the control fibroblasts 16 ± 5% (mean ± S.D., n = 6) of the newly synthesized collagen was degraded during the 18h incubation. The amount of collagen degradation in the OI cells, expressed as a percentage of control values, is given in Table 1. Collagen degradation was approximately twice the control values in OI 30, 31, 24, 39 and 40 and was increased by a third in OI 26 and 35.

The recoveries of the [^{3}H]hydroxyproline standards added to the ethanol-soluble fractions were 84 ± 7% and to the ethanol-precipitated protein fractions they were 100 ± 10% (mean ± S.D., n = 8). These recoveries were better than those reported by Bienkowski & Engels (1981) who used

Table 1. *Collagen production, secretion and degradation in OI fibroblasts*

Collagenous and non-collagenous protein production was determined by the specific collagenase assay of Peterkofsky & Diegelmann (1971), after labelling confluent cell cultures with [^{14}C]proline for 18h. The incorporation of [^{14}C]proline into protein was corrected for differing cell densities by measurement of the DNA content of the cultures and the synthetic rate of the OI cells was expressed as a percentage of the incorporation in control cells incubated simultaneously. Each value represents the mean of at least four separate determinations. Collagen synthesis was calculated by addition of the values obtained for intact collagen produced and collagen degraded. Values were expressed as a percentage of those determined in control cell cultures. Collagen degradation was measured by the appearance of [^{14}C]hydroxyproline in small peptides soluble in 75% (v/v) ethanol, compared with [^{14}C]hydroxyproline in macromolecular ethanol-precipitated collagen (see the Experimental section for details). Collagen degradation in control fibroblast cultures was $16 \pm 5\%$ (mean $\pm$ S.D., $n = 6$). Collagen secretion was determined by the distribution of collagenase-sensitive [^{14}C]proline-labelled protein between the incubation medium and the cell layer fraction (see the Experimental section for details). The data are expressed as percentages of the secretion in control cell cultures and are the mean of at least four separate determinations. The proportion of collagen secreted into the medium by control cells was $71 \pm 5\%$ (mean $\pm$ S.D., $n = 21$).

Cell line	Collagen degradation (% of control)	Collagen production (% of control)	Collagen synthesis (% of control)	Non-collagen protein production (% of control)	Collagen secretion (% of control)
OI 26	131	50	53	60	51
OI 30	196	127	155	110	79
OI 31	186	82	98	83	63
OI 35	135	86	92	106	47
OI 24	188	75	90	106	67
OI 39	198	83	103	91	76
OI 40	171	46	53	115	57

a more complex set of decolourization and ion-exchange chromatographic procedures.

Production of collagenous and non-collagenous proteins

Since a significant proportion of collagen is rapidly degraded intracellularly, it is necesary to differentiate between collagen synthesis, defined as the total amount of collagen synthesized at the mRNA level, and collagen production, the amount of intact newly synthesized collagen (Baum *et al.*, 1980). Therefore, collagen synthesis was calculated using the values obtained for the amount of molecular collagen produced and the amount of collagen degraded during the incubation period. The production and synthesis of collagen and the production of non-collagenous proteins by OI cells are expressed as percentages of control values in Table 1.

Collagen synthesis was reduced to approx. 50% of the control values in OI 26 and OI 40, and was increased in OI 30. However, collagen synthesis was not specifically reduced in the other OI cells and in most cases the synthesis of collagenous and non-collagenous proteins was not significantly different from that of control cells. One patient, OI 26, showed reduced incorporation of isotope into both collagenous and non-collagenous proteins. The specific activity of the intracellular free proline was measured to determine whether this reduction in proline incorporation was due to decreased availability of [^{14}C]proline for protein synthesis. The intracellular proline specific radioactivity of OI 26 was comparable with that of the control cells (80d.p.m./nmol), suggesting that the reduced incorporation of [^{14}C]proline into protein by OI 26 was due to an overall reduction in synthetic activity.

Collagenous and non-collagenous protein secretion

The control fibroblasts secreted $71 \pm 5\%$ (mean $\pm$ S.D., $n = 21$) of their collagen while the OI fibroblasts only secreted 33–58% of their collagen. The results from the OI cells, expressed as a percentage of control values, are shown in Table 1. Collagen secretion was reduced by approx. 50% in OI 26, 35 and 40, and by 21–37% in the other patients. The secretion of non-collagenous proteins by the OI cells was not significantly different from that of control cells. Both parents of OI 26, 30, 31 and 39 secreted normal amounts of collagen and non-collagenous proteins.

Characterization of the procollagens and collagens

Analysis of the procollagens by electrophoresis showed that both control and OI cell cultures produced pro-α1(I)-, pro-α2(I)-, pro-α1(III)-chains along with their corresponding proteolytic intermediates in the conversion to collagen α-chains. No differences were observed in either the migration or number of chains in the control compared with the OI gels.

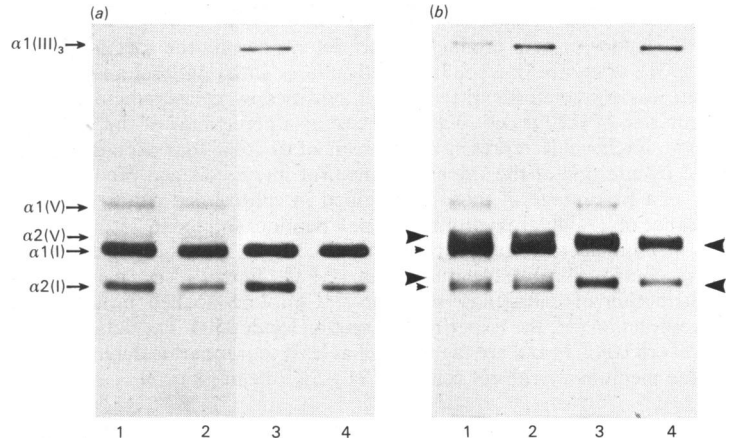

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of the pepsin-digested collagens produced by control and OI fibroblasts*
Cell cultures were labelled with [5-^{3}H]proline and collagens from the cell layer and the incubation medium were subjected to limited pepsin digestion at 4°C for 6h (see the Experimental section for details). The resultant α-chains were analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). (*a*) Collagen from the control cell layer (lanes 1 and 2) and culture medium (lanes 3 and 4). The samples in lanes 2 and 4 were reduced with 10mM-dithiothreitol prior to electrophoresis. (*b*) Collagen from cell layer (lane 1) and medium (lane 2) of OI 26, 30, 31, and 35 and from the cell layer (lane 3) and medium (lane 4) of OI 24, 39 and 40. Samples were electrophoresed without prior reduction. A representative result from each group is presented and the electrophoretic migration of the α-chains of type I, type III and type V collagen are indicated. The large arrows indicate the abnormally migrating α1(I)′- and α2(I)′-chains.

In order to simplify the complex pattern obtained with the procollagens, limited pepsin digestion was performed to convert procollagen, and partially processed procollagens, to collagen. The control cells produced mainly type I collagen and small amounts of type III and V collagens (Fig. 1*a*). Type III collagen was identified by the characteristic migration of the α1(III)-chains before and after reduction of the interchain disulphide bonds. Type V collagen chains were identified by reference to α1(V)- and α2(V)-chain standards and they were characteristically found in the cell layer fractions (Fig. 1*a*, lanes 1 and 2). The proportion of type III collagen produced by both control and OI cells was approx. 7% of the total collagen production with no detectable difference in the proportions of type III collagen secreted. Similar amounts of type V collagen were also produced by control and OI cells (Fig. 1).

The OI cells produced mainly type I collagen but the α-chain patterns obtained by electrophoresis were abnormal. In one group of patients (OI 26, 30, 31, and 35) the collagens obtained from both the cell layer and medium showed normally migrating α1(I)- and α2(I)-chains as well as slowly migrating chains designated α1(I)′ and α2(I)′ (Fig. 1*b*, lanes 1 and 2). In the other group of OI patients (OI 24, 39 and 40) only abnormal α1(I)′- and α2(I)′-chains were observed (Fig. 1*b*, lanes 3 and 4). The parents of OI 26, 30, 31 and 39 however, produced only normal α-chains.

The collagenous nature of α1(I)′- and α2(I)′-chains was verified by their susceptibility to digestion with purified bacterial collagenase. In addition, the conditions used for limited pepsin digestion were varied to ensure that the α1(I)′- and α2(I)′-chains were not artefacts of this procedure. Evidence that these abnormal chains were slowly migrating forms of the α1(I)- and α2(I)-chains was provided by analyses of the α-chains separated by reverse-phase h.p.l.c. (Fig. 2). A representative chromatogram of the collagens obtained from the OI patients whose fibroblasts produced α1(I)′-, α1(I)-, α2(I)′- and α2(I)-chains is shown in Fig. 2(*b*). Electrophoresis of fractions obtained from the heterogeneous α1(I)-chain peak showed that the α1(I)′-chains were eluted first, followed by the normally migrating α1(I)-chains. The α2(I)-chain peak obtained from the chromatogram was also heterogeneous with the α2(I)′-chains eluting before α2(I)-chains (Fig. 2*d*). Furthermore, one-dimensional electrophoresis of the CNBr-cleavage peptides of the purified chains demonstrated that the α1(I)′- and α2(I)′-chains contained the characteristic peptides of α1(I)- and α2(I)-chains, respectively, but all of the peptides migrated more slowly than normal.

The α1(I)′- and α2(I)′-chains from OI 24, 39 and

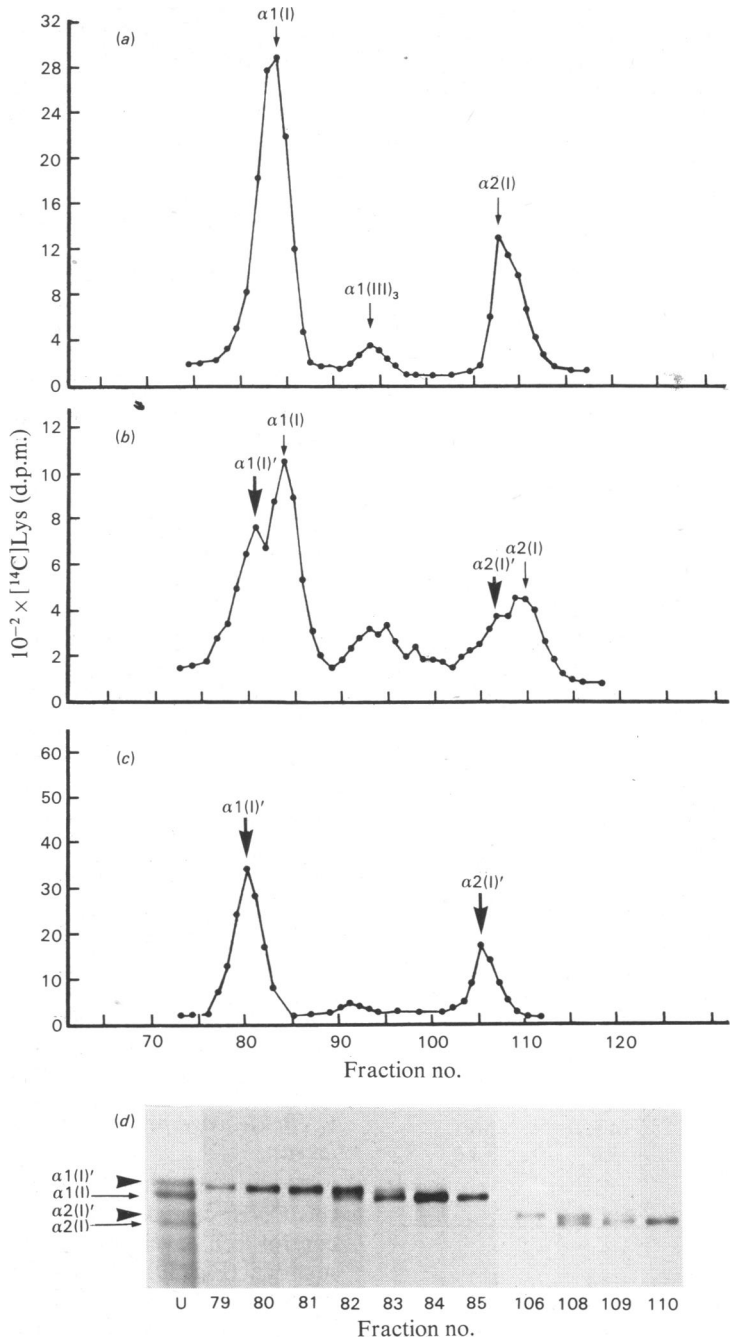

Fig. 2. *Separation of α-chains from control and OI collagens by reverse-phase h.p.l.c.*

[^{3}H]Proline-labelled collagens were denatured and separated by reverse-phase h.p.l.c. with a linear gradient of 12.5% to 37.5% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (see the Experimental section for details). Portions of each fraction were assayed for radioactivity and representative chromatograms of control cells and the two groups of OI cells are shown. (*a*) Control collagen; (*b*) collagen produced by OI 31; (*c*) collagen from OI 40; the elution positions of α1(I)-, α2(I)-chains and $[\alpha 1(III)]_3$ as well as the abnormal α1(I)′- and α2(I)′-chains are indicated; (*d*) portions of the fractions collected from OI 31 were also analysed for collagen α-chain composition by SDS/polyacrylamide-gel electrophoresis. The track designated U is an unfractionated sample of OI 31. The electrophoretic migration of α1(I)-, α1(I)′-, α2(I)-, and α2(I)′-chains is shown.

Table 2. *Lysine hydroxylation in the purified collagen α-chains*

Cell cultures were labelled with [^{14}C]lysine and the collagens were subjected to limited digestion with pepsin. After denaturation the collagen α-chains were purified by h.p.l.c. (see the Experimental section for details). The extent of lysine hydroxylation was determined by ion-exchange chromatographic separation of the [^{14}C]lysine and [^{14}C]hydroxylysine after acid hydrolysis. Values are expressed as the percentage of lysine residues hydroxylated and for control cultures represent the mean ± S.D. of five experiments. The values for the OI cells are the mean of at least duplicate determinations.

		Hydroxylation of lysine (%)			
Cell line	Chain ...	α1(I)	α1(I)′	α2(I)	α2(I)′
Control		34.7 ± 2.8 (5)	–	52.0 ± 1.6 (5)	–
OI 26		36.7	46.6	*	*
OI 30		36.6	41.7	*	*
OI 31		32.6	53.0	46.5	54.6
OI 35		32.9	46.0	50.9	58.8
OI 24		–	51.2	–	60.6
OI 39		–	45.1	–	58.3
OI 40		–	51.3	–	55.5

* Insufficient separation of the α2(I)- from α2(I)′-chains by h.p.l.c. to allow accurate quantification.

Table 3. *Glycosylation of hydroxylysine in purified collagen α1(I)-chains from OI fibroblasts*

The [^{14}C]lysine-labelled α1(I)-chains were purified by h.p.l.c. and subjected to alkaline hydrolysis (see the Experimental section for details). The glycosylation values were determined by quantification of the radioactivity in [^{14}C]lysine-labelled galactosylhydroxylysine (Gal-Hyl) and glucosylgalactosylhydroxylysine (Glc-Gal-Hyl) separated by ion-exchange chromatography. The values were calculated as the number of glycosylated residues per total lysine-plus-hydroxylysine residues determined by amino acid analysis (Kivirikko & Myllylä, 1980).

		No. of residues/35 Lys + Hyl residues	
Cell line	Collagen chain	Gal-Hyl	Glc-Gal-Hyl
Control	α1(I)	4.4	1.6
OI 31	α1(I)	3.9	2.1
	α1(I)′	4.6	6.7
OI 40	α1(I)′	5.6	4.6

40 had similar retention times as the α1(I)′- and α2(I)′-chains from OI 26, 30, 31 and 35 (Fig. 2*c*). One-dimensional electrophoresis of α1(I)′- and α2(I)′-chains from these cells also confirmed that they also contained slower migrating forms of the α1(I)- and α2(I)-chain CNBr-cleavage peptides.

The hydroxylation and glycosylation of the lysine residues was determined in the α-chains prepared by h.p.l.c. (Tables 2 and 3). In control cell cultures, 34.7% of the lysine residues were hydroxylated in the α1(I)-chain and 52% in the α2(I)-chain. The normally migrating α1(I)- and α2(I)-chains from the OI cell cultures had similar hydroxylation values. However, lysine hydroxylation was significantly higher in the α1(I)′-chains than in the α1(I)-chains produced by the same cells or controls. While lysine hydroxylation in the α2(I)′-chain was higher than in normal α2(I)-chains, the increase was not as marked as for the α1(I)′-chains (Table 2). Along with the increased lysyl hydroxylation there was a parallel increase in the extent of hydroxylysine glycosylation in OI 31 and OI 40, representatives of the two groups of OI cells (Table 3). In the α1(I)′-chains from both there was approximately a 3-fold increase in the galactosylhydroxylysine content. The incorporation of radioactivity into the glycosylated derivatives of hydroxylysine in the α2(I)′-chains was insufficient for accurate quantification.

CNBr-cleavage peptides from collagens and procollagens

The two-dimensional electrophoretic 'maps' of the CNBr-cleavage peptides obtained from the control cell layer and medium collagens were identical (Fig. 3*a*) and included the major peptides of the α1(I)- and α2(I)-chains (Cole & Chan, 1981). The peptide 'maps' of the collagens from the group of OI cells displaying α1(I)-, α1(I)′-, α2(I)- and α2(I)′-chains (OI 26, 30, 31 and 35) were similar to each other (Fig. 3*b*) but were different from the control 'maps'. The major difference was in the second dimension, where each peptide was observed to have a slowly migrating and a normally migrating form. This heterogeneity was more marked in the α1(I)- than in the α2(I)-chain cleavage peptides.

The two-dimensional electrophoretic 'maps' of

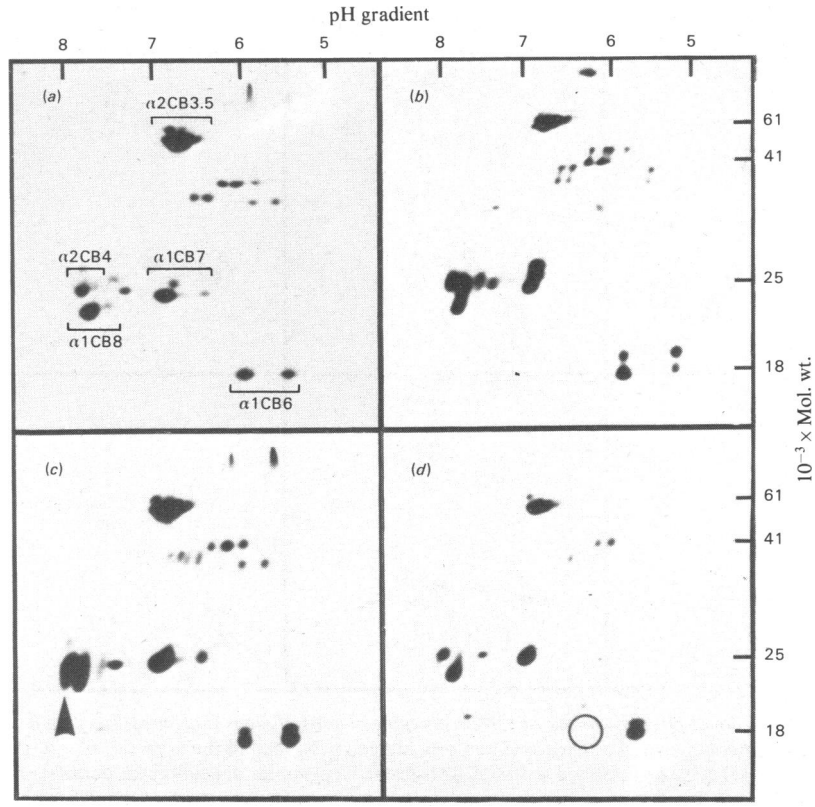

Fig. 3. *Two-dimensional electrophoresis of CNBr peptides of pepsin-digested collagens of OI and control fibroblasts*
CNBr cleavage of pepsin-digested [^{3}H]proline-labelled collagens was achieved in 70% (v/v) formic acid by the method of Cole & Bean (1979). The 'maps' presented are representative of the results obtained for the two groups of OI cells. (*a*) Collagen produced by control cells; (*b*) collagen from OI 26, 30, 31, and 35; (*c*) cell layer collagen of OI 24; (*d*) secreted collagen of OI 24. The open circles indicate the absence of a charged form of α1(I) CB6 and the arrow indicates the presence of a new charged form of the α1(I) CB8 peptide. The peptide maps of OI 24 were otherwise representative of the group producing only abnormal collagen α1(I)′- and α2(I)′-chains (OI 24, 39, 40).

the CNBr-cleavage peptides obtained from the group of OI cells (OI 24, 39 and 40) that produced only α1(I)′- and α2(I)′-chains showed that all the peptides had slower migrations than the peptides from the control cells (Figs 3*c* and 3*d*). Molecular weight heterogeneity was also noted in this group and this was most evident in OI 24, where the peptides from the cell layer and medium showed two closely migrating forms which were more apparent in the smaller molecular weight α1(I)′ CB6 than in the higher molecular weight peptides (Figs. 3*c* and 3*d*). In OI 39 the molecular weight doublets were more distinct in the α1(I)′ CB8 and to a lesser extent in the α2(I)′ CB4 (results not shown). CNBr cleavage of the procollagens resulted in peptide maps similar to those derived from collagen (Fig. 4), with the exception of the apparently increased molecular weights of the α1 CB6 and α2 CB3.5 peptides. Both these peptides are located at the *C*-terminal ends of the α-chains and CNBr-cleavage of procollagen results in the inclusion of some procollagen *C*-terminal propeptide sequences along with the α1 CB6 and α2 CB3.5 peptides. These peptides were thus designated pCα1 CB6 and pCα2 CB3.5 (Fig. 4*a*). The peptides from those cells that produced normal and 'slow' α-chains again resolved into molecular weight doublets (Fig. 4*b*) and the peptides from those OI cells producing only α1(I)′- and α2(I)′-chains showed slowly migrating CNBr-cleavage peptides which also displayed some molecular weight heterogeneity (Fig. 4*c*). The results obtained by 'mapping' unhydroxylated procollagen produced in the presence of α,α′-dipyridyl are shown in Figs. 4(*d*), 4(*e*) and 4(*f*). The peptides of the OI cell collagens (Fig. 4*e*) are indistinguishable from those of the control unhydroxylated procollagen (Fig. 4*d*), with only normally migrating single molecular weight

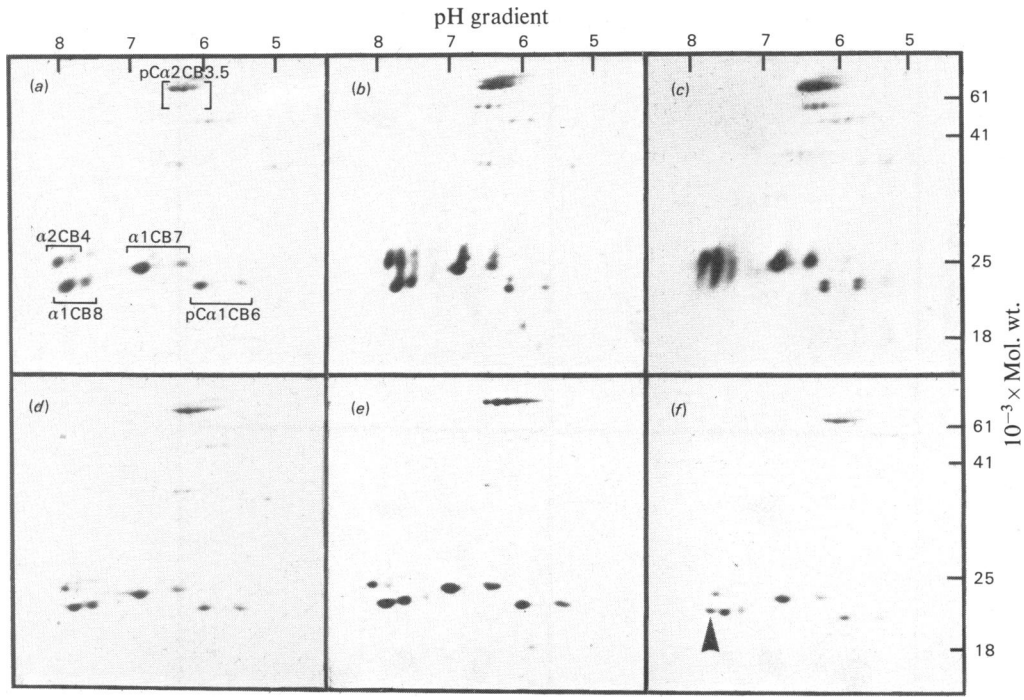

Fig. 4. *Two-dimensional electrophoresis of CNBr peptides of procollagens and unhydroxylated procollagens*
[^{3}H]Proline-labelled procollagen and unhydroxylated procollagen produced in the presence of α,α′-dipyridyl was isolated from control and OI cells and cleaved with CNBr (see the Experimental section for details). The CNBr-cleavage peptides were then analysed by two-dimensional electrophoresis. Representative maps are shown. (*a*) Procollagen from control cells; (*b*) procollagen from OI 26, 30, 31 and 35; (*c*) procollagen from the cell layer of OI 24; (*d*) unhydroxylated procollagen from control cells; (*e*) unhydroxylated procollagen from OI 26, 35, 30 and 31; (*f*) unhydroxylated procollagen from the cell layer of OI 24. The arrow indicates the extra charged form of the α1(I) CB8 peptide. The two unique CNBr-cleavage peptides, which include procollagen sequences along with the characteristic peptides derived from the helical portion of the molecule, are designated pCα1 CB6 and pCα2 CB3.5.

species for each of the peptides. This finding demonstrates that the slow electrophoretic migration of the α1(I)′- and α2(I)′-chains from the OI cells was due to excessive post-translational modification of lysine.

Charge heterogeneity was also noted in some of the OI cell lines. In OI 24 the α1(I) CB8 peptide obtained from the α1(I)′ chain of the cell layer had, in addition to the normal charged components, an extra basic spot (Fig. 3*c*). The extra spot was not observed in the α1(I)′ CB8 peptide of the medium collagen (Fig. 3*d*). It was present in the CNBr-cleavage peptides obtained from the unhydroxylated collagens (Fig. 4*f*), indicating that it was not related to post-translational hydroxylations of lysine or proline.

In OI 24 only one charged form of the α1(I)′ CB6 peptide was detected in the medium (Fig. 3*d*) compared with the two charged forms in the collagens from the cell layer (Fig. 3*c*) and in the collagens from the control cells. The presence of a single charged form of the α1(I)′ CB6 was a variable finding in the other OI collagens as well, and may reflect variations in pepsin digestion of the telopeptide extension of the α1(I) CB6 peptide, since two charged forms of this peptide were always present after CNBr-cleavage of procollagen.

The fluorograms of the CNBr-peptide 'maps' were exposed for prolonged periods to allow examination of the type III and V collagen peptides. In all the OI cells the α1(III) CB5 and the major α1(V)- and α2(V)-chain CNBr cleavage peptides were present as single-molecular-weight, normally migrating forms and with normal charge patterns. These findings indicated that the electrophoretic abnormalities were confined to type I collagen.

Discussion

Cultured dermal fibroblasts from seven consecutive cases of lethal perinatal OI showed increased

collagen degradation, decreased collagen secretion and the production of abnormal α1(I)- and α2(I)-chains of type I collagen. Despite the similarities significant differences were observed in each of these parameters.

The increased levels of collagen degradation and the decreased levels of collagen secretion suggested that the OI cells were producing structurally abnormal collagen. It is likely that the increased collagen degradation occurred within the OI cells, since extracellular collagenases would be inhibited by the serum in the cell culture media (Eisen *et al.*, 1971). Evidence from other studies indicates that a proportion of the newly synthesized collagen is degraded within the cell by a process involving lysosomal enzymes and this degradation is increased when abnormal collagens are synthesized (Berg *et al.*, 1980). The level of intracellular degradation of collagen is also influenced by the culture conditions, but the confluent cultures, optimal levels of prolyl hydroxylation, and the presence of serum used in our study have been shown by Steinmann *et al.* (1981) to minimize the levels of intracellular collagen degradation.

The reduced ability of the OI cells to secrete newly synthesized collagen into the medium was due to a specific defect in collagen secretion rather than to a more generalized transport defect, since the secretion of non-collagenous proteins was normal. However, collagen secretion, which was measured by comparison of the amount of molecular collagen in the cell layer with that in the medium over an 18h period, did not take into account the amount of collagen degraded. In those OI cells with greatly increased collagen degradation this will presumably selectively lower the proportion of the collagen determined in the cell layer, resulting in an over-estimate of collagen secretion.

The electrophoretic studies provided evidence that abnormal type I collagen was produced by the OI fibroblasts. Slowly migrating α1(I)'- and α2(I)'-chains were produced by all OI fibroblasts, which could be classified into two groups on the basis of whether normal collagen α1(I)- and α2(I)-chains were also produced. One group (OI 26, 30, 31 and 35) produced both normal and abnormal α-chains, whereas in the second group (OI 24, 39 and 40) only α1(I)'- and α2(I)'-chains were evident.

The electrophoretic detection of apparent molecular weight heterogeneity was dependent on whether procollagen, collagen or CNBr-cleavage peptides were examined. The procollagen patterns were complex, but they did not reveal any abnormal bands or abnormal migrations. This is in contrast with the report by Barsh & Byers (1981), of a more rapidly migrating pro-α1(I)-chain in a baby with the lethal form of OI. However, abnormalities of migration were detected in all of our cases when the procollagens in our study were examined after removal of the propeptides and telopeptides by limited pepsin digestion. The molecular weight heterogeneity became more apparent when the CNBr cleavage peptides obtained from either procollagen or collagen were examined by electrophoresis, reflecting the increased sensitivity of this technique in detecting migration differences in smaller molecules.

The α1(I)'- and α2(I)'-chains in both groups were shown to contain higher levels of hydroxylysine than the normal α1(I)- or α2(I)-chains from either control cells or the first group of OI patients (OI 26, 30, 31 and 35). The increase in lysine hydroxylation in the α1(I)'- and α2(I)'-chains was particularly significant because of the already high levels in control α1(I)-chains (35%) and α2(I)-chains (52%), as previously reported for cultured fibroblasts (Myllylä *et al.*, 1981). The relative increase in hydroxylation is less marked in the α2(I)'-chain than in the α1(I)'-chain and this may indicate that in cell culture the lysine in the α2(I)-chain is close to maximally hydroxylated. This may account for the greater retardation of migration noted for the α1(I)'-chain than for the α2(I)'-chain. The slower migration of all the major CNBr-cleavage peptides indicated that the increased level of lysine hydroxylation is distributed along the α-chains. The α1(I)'-chain also contained higher than normal levels of glycosylated hydroxylysine. The levels of glucosylgalactosylhydroxylysine were preferentially increased in the OI collagens with only slightly increased levels of galactosylhydroxylysine.

The mechanism underlying the increased post-translational modifications of lysine in the α1(I)'- and α2(I)'-chains is uncertain. One possibility is that triple helix formation by the pro α-chains was delayed, enabling the hydroxylating and glycosylating enzymes to act for a prolonged period. Retarded helix formation has been shown to result in increased lysine hydroxylation and glycosylation, as well as reduced collagen secretion (Prockop *et al.*, 1976; Oikarinen *et al.*, 1976, 1977), and increased degradation (Steinmann *et al.*, 1981). The glucosylgalactosylhydroxylysine content has been shown to be more affected than galactosylhydroxylysine by the retardation of helix formation (Oikarinen *et al.*, 1977). Proline hydroxylation, which is also terminated by the formation of the triple helix, was not increased in the OI cells, presumably because hydroxylation of available proline residues is normally almost complete. A reduced ability to form a normal helix may result from underlying structural defects in the pro-α-chains which prevent normal chain association or initiation of triple helix formation.

Evidence of a possible structural abnormality was found in the α1(I)′ CB8 peptide obtained from OI 24, which contained an additional basic component when examined by two-dimensional electrophoresis. This abnormality was seen in the peptide maps of the unhydroxylated procollagens, demonstrating that it was not due to post-translational hydroxylation differences. It was also present in the purified α1(I)-chains obtained from the dermis, calvarium and femur of this baby (J. F. Bateman, T. Mascara, D. Chan & W. G. Cole, unpublished work). It is unclear whether this finding represents an amino acid sequence polymorphism or is related to OI.

Since OI 26, 30, 31 and 35 produced α1(I)′-, α2(I)′-, α1(I)- and α2(I)-chains, it is likely that these cells were producing two forms of the type I collagen molecule. The normally migrating chains possibly exist in a set of molecules which are secreted normally and the slowly migrating chains in another set of collagen molecules which have reduced secretion and increased degradation. As the fibroblasts from the parents showed only normal α-chains it is likely that this group of patients were heterozygous for a new mutation involving the α1(I)- or α2(I)-chain. In OI 24, 39 and 40 only the abnormal α1(I)′- and α2(I)′-chains were detected, although CNBr cleavage 'maps' showed some apparent molecular weight heterogeneity. The findings would be consistent with a homozygous or double heterozygous defect in either the α1(I)- or α2(I)-chain. Only the parents of OI 39 have been studied and no abnormalities were detected.

The collagen abnormalities noted in our seven OI patients appear to be specific for type I collagen, as type III and type V collagens had normal electrophoretic mobilities. There was no evidence of an increase in the amount of type III collagen or type V collagen (Penttinen *et al.*, 1975; Pope *et al.*, 1980).

The findings in our study suggest that the decreased secretion of collagen, increased degradation of collagen, and synthesis of α1(I)′- and α2(I)′-chains with increased lysyl hydroxylation and glycosylation are common features in the lethal perinatal form of OI. Recent studies on collagen from the tissues of two OI patients also reported increased lysyl hydroxylation and glycosylation (Trelstad *et al.*, 1977; Kirsch *et al.*, 1981). Further delineation of these defects should provide information concerning the mechanisms involved in producing the severe bone fragility in the lethal perinatal form of OI and should also provide an insight into the normal processes of collagen chain assembly, post-translational hydroxylation and glycosylation, intracellular degradation, and secretion.

We thank Professor D. M. Danks, Dr. J. Rogers, Dr. P. Campbell and Professor D. Sillence for their assistance. This work was supported by grants from the National Health and Medical Research Council of Australia, the Clive and Vera Ramaciotti Foundation, the Utah Foundation and the Australian Orthopaedic Association.

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