Substitution of aspartic acid for glycine at position 310 in type II collagen produces achondrogenesis II, and substitution of serine at position 805 produces hypochondrogenesis: analysis of genotype–phenotype relationships

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Two different mutations were found in two unrelated probands with lethal chondrodysplasias, one with achondrogenesis type II and the other with the less severe phenotype of hypochondrogenesis. The mutations in the *COL2A1* gene were identified by denaturing gradient gel electrophoresis analysis of genomic DNA followed by dideoxynucleotide sequencing and restriction site analysis. The proband with achondrogenesis type II had a heterozygous single-base mutation that substituted aspartate for glycine at position 310 of the $\alpha 1(\text{II})$ chain of type II procollagen. The proband with hypochondrogenesis had a heterozygous single-base mutation that substituted serine for glycine at position 805. Type II collagen extracted from cartilage from the probands demonstrated the presence of type I collagen and a delayed electrophoretic mobility, indicating post-translational overmodifications. Analysis of CNBr peptides showed that, in proband 1, the entire peptides were overmodified. Examination of chondrocytes cultured in agarose or alginate

indicated that there was a delayed secretion of type II procollagen. In addition, type II collagen synthesized by cartilage fragments from the probands demonstrated a decreased thermal stability. The melting temperature of the type II collagen containing the aspartate-for-glycine substitution was reduced by 4 °C, and that of the collagen containing the serine-for-glycine substitution was reduced by 2 °C. Electron microscopy of the extracellular matrix from the chondrocyte cultures showed a decreased density of matrix and the presence of unusually short and thin fibrils. Our results indicate that glycine substitutions in the N-terminal region of the type II collagen molecule can produce more severe phenotypes than mutations in the C-terminal region. The aspartate-for-glycine substitution at position 310, which was associated with defective secretion and a probable increased degradation of collagen, is the most destabilizing mutation yet reported in type II procollagen.

INTRODUCTION

Hypochondrogenesis is a lethal chondrodysplasia that appears to represent a mild form of achondrogenesis type II [1,2]. However, there may well be phenotypic overlap between these chondrodysplasias and the non-lethal chondrodysplasia spondyloepiphyseal dysplasia congenita, since a continuous gradient of severity was seen in affected individuals, thus supporting the concept of a chondrodysplasia family [3]. The hypothesis that these chondrodysplasias are closely related is substantiated by the recent demonstrations that heterozygous mutations in the *COL2A1* gene for type II collagen are found in probands with all three phenotypes [4–11]. With most chondrodysplasias it has been difficult to establish the relationship between the genetic defect and the clinical phenotype because cartilage is not available from many probands, and chondrocytes are difficult to maintain in culture without dedifferentiation [12]. New techniques for culturing chondrocytes in three-dimensional systems, however, have been useful to either maintain the chondrocyte phenotype or restore the phenotype after increasing the cell number [7,9,13].

Here we have identified a mutation in the *COL2A1* gene in one proband with hypochondrogenesis and in another proband with achondrogenesis type II. In addition, by using explant and chondrocyte cultures we have analysed the consequences of the mutations on the function of the protein and the morphology of the tissues.

MATERIALS AND METHODS

Case reports

Severe dwarfism was detected by ultrasound examination of the two fetuses at 33 and 35 weeks of gestation. Clinical and radiological examinations of proband 1, who died at birth, were consistent with the diagnosis of hypochondrogenesis, whereas proband 2 was diagnosed as having achondrogenesis type II after termination of pregnancy at 34 weeks. Both had short stature, short limbs and macrocephaly (cranial perimeter of proband 2 was 34 cm at 34 weeks). Also, proband 1 had a median cleft palate and anteverted nares. Both probands had short ribs and

Abbreviations used: DGGE, denaturing gradient gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; β APN, amino propionitrile; RER, rough endoplasmic reticulum.

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Figure 1 X-rays and histopathology

(a) Radiograph of proband 1 (hypochondrogenesis) at 34 weeks; (b) radiograph of proband 2 (achondrogenesis II) at 33 weeks; (c) histopathology of growth plate cartilage in proband 2 showing hypercellularity with irregular chondrocyte columns and vascular penetration.

flat and poorly ossified vertebral bodies, mostly in the sacrum and cervical regions (Figures 1a and 1b). The long bones were short with cupped metaphyses, especially in the proband with achondrogenesis type II, and the humeral metaphyses were markedly enlarged. The iliac wings were hypoplastic and the pubic branches were not visible. The shortening of the long bones and the femoral cupping were more pronounced in proband 2 than in proband 1. Histological examination of articular cartilage from proband 2 showed abundant cartilage canals, a higher cellularity than in control cartilage and an abnormal line of ossification with disorganized chondrocyte columns (Figure 1c). In both cases, parents were unrelated, phenotypically normal and had no other children.

DNA extraction and denaturing gradient gel electrophoresis (DGGE) analyses

Genomic DNA was isolated either from confluent fibroblast cultures or from peripheral blood by conventional methods [14] and used for PCR amplification. All 44 exons of the *COL2A1* gene coding for the helical domain of type II collagen and corresponding exon-flanking sequences were amplified by PCR and analysed by DGGE. The sequences of the primers and the conditions for amplification and DGGE analysis were reported previously [15].

Sequencing and restriction site analysis

For direct sequencing of the PCR product containing exon 42 from proband 1, single-stranded DNA was generated by two rounds of PCR amplification. Double-stranded DNA was first

Table 1 Primers used for direct sequencing and restriction mapping

		Location	Size (bp)
Exon 42	Primer 1 GTGCTTACCACTTGCACTCA	Intron 41	206
	Primer 2 ACTCACAGGGCCCTCTCCC	Intron 42	
	Primer 3 GGGGTTGGTGGGTTAGTGGG	Intron 21	
Exon 22	Primer 4 CCTGGCAGTGCAGGGCTGGA	Intron 23	457

synthesized by using primers 1 and 2 (Table 1). A 5 μ l aliquot was used for re-amplification with only one primer. The product was purified on a Centricon 100 column (Amicon Corp.) and sequenced with a Sequenase kit (USB). The 206 bp PCR fragment corresponding to exon 42 (amplified with primers 1 and 2) from proband 1 and his parents was digested with *HpaII* and *MspI*, then electrophoresed on a 2.5 % Nu Sieve (FMC) agarose gel stained with ethidium bromide. The DNA fragment covering exons 22 and 23 of proband 2 was amplified by using primers 3 and 4 (Table 1). The 457 bp fragment was cloned into a *SmaI*-cut and dephosphorylated Bluescript KS vector. Eight positive clones were selected for the preparation of double-stranded DNA, which was purified on Quiagen columns (Diagen) and then sequenced with Sequenase 2.0 in both directions.

Explant and chondrocyte cultures

Femoral articular cartilage was dissected free of perichondrium and bone in sterile PBS and finely diced into 0.1 mm³ pieces for explant and cell cultures. To prepare explant cultures, thin slices of cartilage (100–500 μ m) were preincubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS) and 50 μ g/ml ascorbic acid for 4 h at 37 °C. This medium was replaced by 2 ml of incubation medium depleted in FCS but containing ascorbic acid, amino propionitrile (β APN) (50 μ g/ml each) and [¹⁴C]proline (10 μ Ci/ml). The medium was replaced after 48 h and the incubation was continued for 2 days. Cartilage fragments and pooled media were harvested and treated separately.

For chondrocyte cultures, cells were released by sequential enzymic digestion with 0.05 % hyaluronidase (Sigma), 0.01 % trypsin and 0.1 % bacterial collagenase (Boehringer Mannheim) as described previously [16]. Cells were washed in DMEM supplemented with 10 % FCS, 500 units/ml penicillin and 200 μ g/ml streptomycin. The cells were then seeded in the same medium at a density of 2.5×10^4 cells/cm² in plastic flasks. They were either trypsin-treated and passaged once or twice, or used directly for redifferentiation in agarose or in alginate beads. For growth in agarose, chondrocytes were resuspended at a density of 4×10^6 cells/ml in 0.4 % low-melting agarose and grown for 12–15 days in DMEM containing 10 % FCS [12]. Cultures in alginate beads followed the conditions described by Häuselmann et al. [17]. After trypsinization, chondrocytes were washed twice with sterile 0.15 M NaCl solution, then suspended in a mixture of sodium alginate solution and DMEM at a density of 3.4×10^6 cells/cm². The mixture was poured into a 10 ml plastic syringe equipped with a 20-G needle. Polymerization occurred by dropping the mixture into 40 ml of a 102 mM CaCl₂ solution. After 10 min the CaCl₂ solution was decanted and the beads were

washed three times in sterile 0.15 M NaCl solution and once in DMEM. Beads (100–150) were placed at 37 °C in T75 flasks containing 30 ml of DMEM/FCS/antibiotics. Medium was changed every 3 days and cells were cultured for 15–20 days.

Isolation of collagen

Collagens from the cartilage fragments were prepared as described previously [16]. Briefly, the cartilage was homogenized and extracted in 1 M NaCl, 50 mM Tris/HCl, pH 7.4, containing proteinase inhibitors for 48 h at 4 °C under constant stirring. The soluble fraction contained the neutral-salt-soluble collagen. After removal of the proteoglycans with 4 M guanidinium/HCl, 50 mM Tris/HCl, pH 7.5, for 48 h at 4 °C, the insoluble residue was extensively washed with cold water and digested with pepsin in acetic acid for 24 h at 4 °C. Type II collagen was partially purified by precipitation with 0.8 M NaCl in 0.5 M acetic acid. Redifferentiated chondrocytes grown for 20 days in agarose or in alginate beads were incubated for 48 h with [¹⁴C]proline (10 µCi/ml) in 10 ml of serum-free DMEM supplemented with ascorbic acid and βAPN (50 µg/ml). The culture medium was collected, and the alginate beads or agarose were washed with 5 ml of saline solution which was then added to the culture medium. Collagenous proteins secreted into the medium were precipitated by ammonium sulphate (180 mg/ml) followed by pepsin digestion for 2 h at room temperature in 0.5 M acetic acid. Agarose was scraped and resuspended in saline solution, then homogenized with a Polytron and centrifuged at 30 000 *g* for 1 h. The pellet was resuspended in 0.5 M acetic acid and submitted to pepsin digestion.

The alginate beads were disrupted by treatment for 10 min with 50 mM EDTA until complete dissolution. The cells were pelleted at 1200 rev./min (800 *g*) for 10 min and washed twice with 0.15 M NaCl supplemented with proteinase inhibitors. Cells were lysed with 0.1 % Nonidet P40 in 50 mM Tris/HCl, pH 7.5. The cell lysate was treated with pepsin to prepare collagen chains corresponding to the intracellular and pericellular fractions. To examine collagen synthesis and secretion, 5 × 10⁶ cells were cultured for 20 days and labelled with 200 µCi of L-[4,5-³H]proline (30 Ci/mmol) for 24 h. In alginate, three different fractions were harvested separately: the culture medium, the cell pellet and the alginate/EDTA mixture (supernatant). In agarose, collagen synthesis was measured in the culture medium and the cell/agarose fraction. Samples were dialysed extensively against acetic acid solution, followed by hydrolysis with 6 M HCl at 110 °C for 24 h. Tritiated hydroxyproline was separated from proline by passage through an ion-exchange resin as described previously [18].

Quantification of collagen in cartilage

Total collagen content in cartilage hydrolysates was estimated by the colorimetric determination of hydroxyproline amount according to the method of Stegemann [19]. Results were expressed as mg of collagen per g of dry cartilage.

Electrophoresis of collagen chains and CNBr peptides

Neutral-salt- and pepsin-soluble collagens isolated from cartilage or cultured chondrocytes were electrophoresed on 5 % polyacrylamide gels using the discontinuous buffer system of Neville and Glossman [20]. Gels were successively stained with Coomassie Blue and processed for fluorography. For peptide analysis, collagen chains obtained by precipitation with 0.8 M NaCl were cleaved with CNBr. The resulting peptides were separated on 12 % polyacrylamide/SDS gels.

Thermal stability

Thermal denaturation studies were performed by using the proteinase digestion technique of Bruckner and Prockop [21]. Aliquots of pepsin-soluble collagen extracted from the cartilage of proband 1 and [¹⁴C]collagen secreted into the culture medium of tissue from controls and proband 2 were heated from 35 °C to 44 °C and digested with trypsin for 2 min. The reaction was blocked with soybean trypsin inhibitor. Samples were boiled for 3 min in the presence of loading buffer (30 % glycerol, 10 % SDS, 0.01 % Bromophenol Blue in 125 mM Tris/HCl, pH 6.8) and electrophoresed.

Electron microscopy

Alginate beads were fixed with glutaraldehyde (2.5 %) in cacodylate buffer containing sucrose and CaCl₂ (20 mM). Post-fixation was in 1 % OsO₄ before embedding in epon. Agarose pieces were treated similarly but the fixative solution was devoid of CaCl₂ [16]. Semi-thin sections were stained with Toluidine Blue and examined by light microscopy. A Zeiss electron microscope was used for examination of thin sections stained with uranyl acetate and lead citrate.

RESULTS

DGGE analysis of mutations

A total of 44 exons of the *COL2A1* gene and the corresponding exon–intron boundaries were analysed by DGGE for sequence variations. In addition to previously reported neutral polymorphisms [15], DGGE identified two additional sequence variations which were absent from control samples. The variations were detected in PCR products covering exon 42 of

Figure 2 Detection of mutations in exons 42 (a) and 22–23 (b) by DGGE analysis

(a) An additional band (arrow) is observed in lane P (proband 1). (b) An additional band (arrow) is observed in lane 2 (proband 2). The other lanes correspond to control DNA or DNA from individuals with different chondrodysplasias.

Figure 3 Direct sequencing of genomic DNA amplification product containing exon 42

(a) The G→A transition is shown by an arrow. The glycine codon at position 805 is converted into serine. A disrupted *HpaII* site is underlined. (b) *HpaII* (lanes 1–3) and *MspI* (lanes 4–6) restriction analysis of amplified genomic DNA from proband 1 (lanes 2 and 5) and his parents (father, lanes 1 and 4; mother, lanes 3 and 6).

proband 1 (Figure 2a) and exons 22–23 of proband 2 (Figure 2b). Exons 22 and 23 were amplified in the same PCR reaction, since intron 22 was sufficiently small to be amplified together with the flanking exons.

Sequencing of the PCR products corresponding to exons 42 and 22–23 revealed a single base substitution in each case, that converted glycine into serine at position 805 (G→A transition in exon 42) (Figure 3a) and glycine into aspartic acid at position 310 (G→A transition in exon 22) (Figure 4). In exon 42 the base substitution obliterated an *HpaII* restriction site. This property was used to confirm the heterozygosity of the mutation. The mutation was not detectable in the parental leukocytes (Figure 3b), suggesting that it was sporadic.

Quantification of collagen in cartilage

The collagen content of cartilage samples from both probands and controls were measured. Proband 2 presented a dramatic reduction in collagen content (94 mg/g of dry cartilage versus 325 ± 50 mg/g for controls; $n = 7$), whereas proband 1 was not significantly different from control (280 mg/g).

Electrophoretic studies of collagens

The cartilage explant system was used to analyse simultaneously the resident collagens of cartilage and the newly synthesized molecules (^{14}C -labelled).

Figure 4 Sequences of normal and mutant DNA clones obtained by cloning of a 457 bp PCR product

The circle indicates the base substitution which converted Gly-310 into Asp.

Resident collagens

Collagens extracted sequentially in neutral salt buffer and after pepsin digestion were analysed by gel electrophoresis. In the neutral-salt collagen fraction, an $\alpha 1(\text{II})$ chain with normal mobility was predominant. In addition, in both probands a faint band with a delayed mobility was detectable (Figure 5a). In the pepsin-soluble collagen fraction a broad band corresponding to the juxtaposition of two components was observed (Figure 5b). The lower part migrated as the control $\alpha 1(\text{II})$ chain, whereas the

Figure 5 Electrophoretic studies of collagen chains extracted from control (C) and proband (P1, P2) cartilage

(a) Neutral-salt-soluble collagen; (b) pepsin-soluble collagen (PSC); (c) 0.8 M NaCl-precipitated fraction of PSC. Gels were stained with Coomassie Blue. * indicates an $\alpha 1(\text{II})$ chain with decreased mobility.

Figure 6 CNBr peptide analysis of pepsin-solubilized type II collagen from control and proband cartilage

Peptides were resolved on 12% polyacrylamide gels. Gels were stained with Coomassie Blue. (a) Lane 1, control type I collagen; lane 2, mixture of type II and type I collagen (ratio 4:1); lane 3, proband 1 collagen; lane 4, control type II collagen. The arrowheads in lane 3 indicate peptides with delayed mobility. (b) Lane 5, control 1 cartilage; lane 6, proband 1 cartilage; lane 7, control 2 cartilage; lane 8, proband 2 cartilage. Type I collagen CB peptides were derived from the $\alpha 1(I)$ chain (CB 7, 8, 6 and 3) and the $\alpha 2(I)$ chain (CB 4 and 3/5). The additional band between $\alpha 1(I)$ CB6 and $\alpha 1(II)$ CB11 was not seen consistently (lanes 5, 7 and 8) and is likely to represent a non-specific peptide of non-collagenous origin.

Figure 7 Electrophoretic analysis of newly synthesized [^{14}C]proline-labelled collagen extracted from cartilage in explant culture or from chondrocytes cultured in agarose

In explant culture, culture medium (CM) and cartilage fragments (tissue) were studied separately. The neutral-salt-soluble fractions (lanes 1–8) were compared with pepsin-treated material (lanes 9 and 10). The collagen precursors $\text{pro}\alpha 1(II)$ and $\text{p}\alpha 1(II)$ were detected after reduction by dithiothreitol (DTT) treatment (lanes 1–4, 7 and 8). In agarose, the matrix elaborated by control (C) and proband 2 (P2) chondrocytes was pepsin-treated (lanes 11 and 12). The asterisk indicates an $\alpha 1(II)$ chain with decreased mobility.

upper part co-migrated with the $\alpha 3(XI)$ chain. It was assumed to contain both $\alpha 3(XI)$ and overmodified $\alpha 1(II)$ collagen chains. When type XI collagen was eliminated by sequential NaCl precipitation, the 0.8 M NaCl collagen fraction from both probands still exhibited a broad $\alpha 1(II)$ band (Figure 5c). An additional band with the same mobility as the $\alpha 2(I)$ chain of collagen type I was also detected in the probands' material. The presence of collagen type I was supported by the detection of $\beta 1-2$ dimers. Since the $\alpha 1(I)$ chain of collagen type I co-migrates with the $\alpha 1(II)$ chain, the broad $\alpha 1(II)$ chain seen in probands is likely to represent a mixture of three components [normal and overmodified $\alpha 1(II)$ chains, and $\alpha 1(I)$ chains].

CNBr-derived peptides (designated CB peptides) derived from

Table 2 Collagen synthesis by chondrocytes cultured in alginate beads

Chondrocytes in alginate beads (6×10^6 cells/flask) were cultured for 23–25 days and then labelled for 24 h with 200 μl of [^3H]proline. Radioactivity was measured in the medium, the alginate/EDTA mixture and the cells themselves. The total amount of hydroxyproline was then calculated. Values in parentheses represent the percentage of hydroxyproline compared with the control (taken as 100%).

	$10^{-3} \times [^3\text{H}]\text{Hydroxyproline}$ (d.p.m./ 10^6 cells)				Collagen type II/XI*
	Medium	EDTA	Cells	Total	
Control	30.6	29.2	5.7	65.5	13.8
Proband 1	4.0	15.5	10.6	30.1 (46%)	n.d.
Proband 2	10.6	19.5	3.5	33.6 (51%)	4.7

* The relative intensities of the bands were measured on the autoradiograph by scanning densitometry.

pepsin-soluble collagen (0.8 M NaCl fraction) were run on a 12% polyacrylamide gel. In material from proband 1, peptides CB10.5, CB8 and CB9.7 had a delayed mobility when compared with the control (Figure 6a), suggesting that overmodifications extended beyond the mutation. The presence of an additional peptide which co-migrated with $\alpha 1(I)$ CB6 confirmed that both type I and II collagens were present. Peptides $\alpha 1(I)$ CB8 and CB7 are likely to co-migrate with the $\alpha 1(II)$ CB11 normal and overmodified peptides respectively (Figure 6a). In material from proband 2, the presence of $\alpha 1(I)$ CB peptides was also detected, but the mobility of peptides $\alpha 1(II)$ CB10.5, CB8 and CB9.7 was not clearly different from the control (Figure 6b).

In vitro collagen synthesis

Newly synthesized collagens in cartilage fragments cultured in DMEM in the presence of [^{14}C]proline were detected mainly in the salt-soluble fraction isolated from the labelled tissue. A small amount of material was secreted into the culture medium. Under reducing conditions, three major bands were detected in the medium. The most intense band corresponded to the $\alpha 1(II)$ chain and the two minor upper bands represented procollagen $\text{pro}\alpha 1(II)$ and the partially processed chains $\text{p}\alpha 1(II)$. All these bands in cartilage from the probands had a delayed mobility when compared with control cartilage (Figure 7, lanes 3–8). When the neutral-salt-soluble fraction was pepsin-digested, type XI collagen chains became detectable; their electrophoretic mobilities were normal (Figure 7, lanes 9 and 10). However, no $\alpha 2(I)$ chain was visible. Type II collagen migrated as a broad band with a delayed mobility. The normal $\alpha 1(II)$ collagen, which corresponded to the lower part of the band, was less abundant than the upper part [overmodified $\alpha 1(II)$ chains]. These results, which strictly differed from those obtained with the resident material, indicated that most of the newly synthesized $\alpha 1(II)$ homotrimers contained at least one mutant monomer. They also suggested that part of the abnormal type II collagen, instead of being incorporated into resident collagen, was degraded.

Collagen synthesis by chondrocytes cultured in agarose or in alginate

Chondrocytes were first grown in monolayers in order to increase their number, then transferred to agarose or alginate beads and cultured for at least 10 days to induce the re-expression of cartilage-specific collagens (types II and XI). As expected, the

Figure 8 Thermal stability of $[^{14}\text{C}]$ proline-labelled collagens from (a) control and (b) proband 2 cartilage

Cartilage fragments were incubated for 4 days in DMEM supplemented with $[^{14}\text{C}]$ proline. Culture media were collected and pepsin-digested. Collagen chains were submitted to brief proteinase treatment with trypsin. Arrowheads point to the presence of $\alpha1(\text{II})$ chain subfragments.

$\alpha1(\text{II})$ chain synthesized by chondrocytes from proband 2 cultured in agarose migrated more slowly than that from control chondrocytes (Figure 7, lanes 11 and 12). In alginate beads, the amount of collagen synthesized by chondrocytes was measured in the culture medium, the alginate suspension and the isolated cells, since it was possible to separate cells from the loose matrix present between adjacent cells. In the agarose system, only the

culture medium and the agarose-cell mixture were recoverable separately. The $[^3\text{H}]$ hydroxyproline content was measured in order to evaluate total collagen synthesis. Although variations existed between the different fractions, the total amount of hydroxyproline in proband chondrocytes was markedly reduced (by approx. 50%) when compared with normal chondrocytes (Table 2). In proband 2, the collagen type II/type XI ratio was decreased by about 65% (Table 2), suggesting that the mutation affected mostly type II collagen secretion.

Thermal stability

The thermal denaturation temperature (T_m) of the triple helix of pepsin-digested collagen extracted from cartilage was measured to evaluate the stability of normal and mutant $\alpha1(\text{II})$ chains. Cartilage fragments were incubated with $[^{14}\text{C}]$ proline and the thermal stability was assayed by brief proteinase digestion [21]. In a control sample, 50% of the type II collagen was still resistant to proteolysis at 42 °C, whereas the T_m of the over-modified $\alpha1(\text{II})$ chains was reduced to 40 °C in the collagen sample from proband 1 (results not shown). In the achondrogenesis II sample, the newly synthesized $\alpha1(\text{II})$ chain was already partially cleaved at 35 °C (Figure 8). A fragment of about 65–70 kDa was detectable. When temperature was raised to 38–39 °C the uncleaved $\alpha1(\text{II})$ chain disappeared totally and a second subfragment of about 40 kDa became visible. It co-migrated with another peptide (presumably from type IX collagen). The larger subfragment was digested by proteinases at 40 °C. It was noteworthy that the $\alpha1(\text{XI})$ and $\alpha2(\text{XI})$ chains melted at the expected temperature (39 °C) in samples from both control and proband, indicating that thermal instability was specific for collagen type II. Unfortunately, the amount of material that was available from proband 2 was too small to perform further studies on purified type II collagen at temperatures below 35 °C.

Ultrastructural studies of collagen fibrils in three-dimensional cell cultures

Chondrocytes grown in agarose or in alginate for 10 days were examined by electron microscopy. Cells exhibited a well-

Figure 9 Electron micrographs of chondrocytes cultured in agarose for 10 days

Ultrastructure of control chondrocyte (a) and proband 1 chondrocyte (b). A prominent Golgi apparatus (G) was present in both cells. The RER (R) was more visible in the proband cell. Bars = 2 μm.

Figure 10 Electron micrographs of the extracellular matrix produced by chondrocytes in agarose

The matrix produced by control cells (a) contained abundant collagen fibrils (F). A decreased amount of matrix and short fibrils were visible in matrix produced by cells from probands 1 (b) and 2 (c). Bars = 250 nm.

developed rough endoplasmic reticulum (RER) and an abundant Golgi system (Figures 9a and 9b). However, in contrast to previous *in situ* studies, there was no evidence of large vacuoles of dilated RER in proband chondrocytes (see [11,22]). A marked decrease in the extracellular matrix density was observed. Although variability existed between different fields, most of the collagen fibrils in both probands looked less abundant and shorter than in control cells (Figures 10a–10c). These observations were consistent with the decreased collagen secretion by proband chondrocytes.

DISCUSSION

We have identified a single-base heterozygous mutation in the *COL2A1* gene in one proband with hypochondrogenesis, and another single-base heterozygous mutation in the same gene in another proband with achondrogenesis type II. One mutation substituted a serine codon for a glycine codon, and the other an aspartic acid codon for a glycine codon. Since the collagen triple helix folds in a C- to N-terminal direction, it has been suggested that mutations in the C-terminal domains are more likely to produce severe phenotypes than mutations in the N-terminal domains (see [23]). The results here are inconsistent with this general conclusion, since the substitution of aspartic acid for glycine-310 produced a more severe phenotype than the substitution of serine for glycine-805. To the best of our knowledge, the substitution of glycine-310 is the most N-terminal amino acid substitution reported in achondro-hypochondrogenesis [4,6,7,10,11]. In contrast, most reported mutations in the N-terminal domain of the type II collagen triple helix were found in non-lethal chondrodysplasias such as spondylo-epimetaphyseal dysplasia [24], Kniest dysplasia [25] and Wagner–Stickler syndrome [26]. The more severe phenotype seen here may be explained by the nature of the amino acid substitution, since all aspartate-for-glycine substitutions in the *COL1A1* and *COL1A2* genes produce lethal osteogenesis imperfecta [23,27]. It is also possible, however, that the specific sequence at the aspartate-to-

glycine substitution is critical, since the mutation changed the normal sequence Glu-Arg-Gly to Glu-Arg-Asp. The presence of a second negatively charged residue in the sequence may well destabilize the triple helix since, as suggested by Katz and David [28], intrachain salt linkage formation may contribute to the normal stability of the collagen triple helix.

The substitution of aspartate for glycine-310 markedly reduced the stability of the triple helix, as assayed by brief proteinase digestion. The presence of aspartate may well have caused local unfolding of the triple helix, making the adjacent arginine susceptible to trypsin digestion. The marked destabilization of the triple helix caused by the aspartate-for-glycine substitution, however, is somewhat surprising, since reductions in thermal stability were not seen in recombinant type II procollagen with an internal deletion of 12 exons covering much of the same region of the molecule [29]. Also, no reduction in thermal stability was seen with substitutions in the coding sequences of type II procollagen in achondro-hypochondrogenesis or spondyloepiphyseal dysplasia [9,11,30]. The aspartate-for-glycine substitution at position 310 appears to be the most destabilizing mutation reported to date in the type II collagen molecule.

Tissue culture and three-dimensional cultures of chondrocytes were initiated in order to examine the consequences of the mutations on the elaboration of the matrix. Quantification of collagen synthesis indicated that the present substitutions lead to inefficient type II collagen secretion, a result that was supported by electron microscopic examination of the matrix surrounding cultured chondrocytes. In agreement with previous results using chondrocytes on agarose [7], the number of collagen fibrils relative to proteoglycan granules was apparently reduced. Unexpectedly, dilated vacuoles of RER were not seen. The discrepancy between these results and *in situ* findings [11,22] is not clearly understood, but might be related to the *in vitro* culture system itself and/or to a rapid degradation of unstable molecules without intracellular retention. Indirect evidence for increased degradation is provided by the observation that the amount of total resident collagen that was extractable from proband 2

cartilage was reduced by 70 % when compared with the control, whereas the amount of labelled collagen synthesized by chondrocytes in culture was decreased by only 50 %. In addition, the difference between the amount of resident collagen which was rich in normal $\alpha 1(\text{II})$ chains and the newly synthesized material, which contained mostly abnormal chains, supports the hypothesis that a proportion of the abnormal chains were not incorporated into stable fibrils but were rapidly degraded.

Type I collagen is normally absent from control cartilage, but it was previously reported to replace completely type II collagen in two patients with achondrogenesis II [31,32]. The situation was different in our proband 2. Type I collagen is likely to originate from the numerous infiltrating vascular canals, since it was detected at significant levels in the resident collagen but was not synthesized in organ culture. Therefore, in our case, type I collagen did not appear to be the major component of cartilage. Its amount was in the same range in proband 2 as in proband 1, suggesting that there is no strict correlation between the abundance of type I collagen in achondro-hypochondrogenesis and the clinical severity of the disease.

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