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Characterization of a type II collagen gene (*COL2A1*) mutation identified in cultured chondrocytes from human hypochondrogenesis

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ABSTRACT **A subtle mutation in the type II collagen gene *COL2A1* was detected in a case of human hypochondrogenesis by using a chondrocyte culture system and PCR-cDNA scanning analysis. Chondrocytes obtained from cartilage biopsies were dedifferentiated and expanded in monolayer culture and then redifferentiated by culture over agarose. Single-strand conformation polymorphism and direct sequencing analysis identified a G → A transition, resulting in a glycine substitution at amino acid 574 of the proα1(II) collagen triple-helical domain. Morphologic assessment of cartilage-like structures produced in culture and electrophoretic analysis of collagens synthesized by the cultured chondrocytes suggested that the glycine substitution interferes with conversion of type II procollagen to collagen, impairs intracellular transport and secretion of the molecule, and disrupts collagen fibril assembly. This experimental approach has broad implications for the investigation of human chondrodysplasias as well as human chondrocyte biology.**

Chondrogenesis is responsible for the formation of the cartilage templates that serve as temporary models for the vertebrate skeleton. The process is crucial for the development of most embryonic bones and their subsequent linear growth—i.e., endochondral ossification. In both instances, this process involves the biosynthesis, secretion, and assembly of a highly ordered extracellular matrix by differentiated chondrocytes (1). The ability of cartilage matrix to serve its template functions depends on its integrity, which in turn depends on the proper interactions between a three-dimensional network of collagen fibrils comprised of type II collagen and lesser amounts of types IX and XI collagen and a host of other matrix constituents, such as proteoglycans (2).

Abnormalities of chondrogenesis and of the genes expressed during chondrogenesis, especially type II collagen, have long been suspected of causing certain of the human chondrodysplasias. Patients with chondrodysplasias typically have short, deformed bones that grow slowly (3). Their clinical features vary widely and reflect the degree to which endochondral ossification and other normal cartilage functions are disturbed. The candidate disorders for mutations of *COL2A1*, the gene encoding type II collagen, are the spondyloepiphyseal dysplasias (SEDs) (4, 5). In these disorders the distribution of clinical disease parallels the distribution of type II collagen, defective chondrogenesis has been inferred from microscopic studies of growth plate and other cartilages (3, 6–8), and electrophoretic abnormalities of type II collagen have been found (5, 9–11). Recently, reports of *COL2A1* mutations in patients with SED have begun to emerge, validating the earlier suspicions (12–16).

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The detection of mutations in genes expressed during chondrogenesis remains difficult in humans. Cartilage is not easily accessible, and it is relatively acellular. Consequently, it is a poor source of RNA for cDNA synthesis and analysis. Even more problematic has been investigation of the consequences of mutations on chondrogenesis. Despite detailed knowledge of growth plate morphology (17), there is a dearth of biologic information about endochondral ossification in humans. Furthermore, experimental models for studying chondrogenesis in humans are generally not available. Chondrocytes cultured under usual conditions lose their phenotypic characteristics and cease to express many of the genes normally expressed during chondrogenesis (18). Thus, even when a mutation is identified, its biologic significance is difficult to determine.

To circumvent these technical problems and facilitate characterization of chondrogenesis mutations, we developed an experimental approach based on analysis of the products of cultured chondrocytes from affected patients. The plasticity of the chondrocyte phenotype was exploited to expand the population of cultured chondrocytes and to promote chondrogenesis *in vitro*. The mutation was detected by scanning analysis of COL2A1 cDNA derived from reverse-transcribed RNA extracted from cultured chondrocytes, rather than by direct analysis of genomic DNA. We used the approach to identify a *COL2A1* mutation and examine its effects on chondrogenesis in a case of human hypochondrogenesis.

METHODS

Cell Culture. Epiphyseal chondrocytes were obtained from costal cartilage at autopsy from an infant with radiographic characteristics of hypochondrogenesis. Control epiphyseal chondrocytes were obtained similarly from infants who showed no evidence of skeletal abnormalities.

The methods for chondrocyte isolation and monolayer culture have been described in detail (19). Briefly, the cartilage was trimmed to remove perichondrium, minced, and digested overnight with collagenase. The cells were suspended in Dulbecco's modified Eagle's medium/10% fetal calf serum/gentamycin, and primary cultures were established. The cultures were fed twice weekly and passaged two to four times.

Abbreviations: SED, spondyloepiphyseal dysplasia; TEM, transmission electron microscopy; rER, rough endoplasmic reticulum; SSCP, single-strand conformation polymorphism.

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The agarose culture scheme was adapted from that described by Tacchetti et al. (20) for culture of chicken chondrocytes. The cells were trypsinized, and 2×10^6 cells were suspended in 1 ml of medium (Dulbecco's modified Eagle's medium/10% fetal calf serum/gentamycin). The cell suspension was layered over culture dishes precoated with high-temperature agarose. Fresh medium was added every third day. Ascorbic acid at 50 μg/ml was added daily, except for days 1 and 2, when 10 and 25 μg/ml were added, respectively; ascorbic acid was omitted from some cultures. The agarose cultures were monitored daily and maintained for 7–10 days.

Microscopic Analysis. Specimens of costochondral cartilage were dissected and processed into Spurr epoxy resin (Ted Pella) by standard techniques for both light microscopy and transmission electron microscopy (TEM) (19). The tissue-like structures formed in agarose culture were also examined by both light microscopy and TEM. The structures were fixed in 1.5% (wt/vol) paraformaldehyde/1.5% glutaraldehyde/0.05% ruthenium hexamine trichloride/0.1 M sodium cocadylate buffer, pH 7.2. Cultures used for TEM were postfixed in 1.0% (wt/vol) OsO_4. Both were embedded in Spurr epoxy resin. Light microscopy sections were stained with toluidine blue, whereas TEM ultrathin sections were stained with 4% (wt/vol) uranyl acetate/2% (wt/vol) Reynolds lead citrate (Ted Pella).

Collagen Biosynthetic Analysis. Seven- to ten-day cultures were labeled with [^{3}H]proline for 24 hr (20). After being washed, the cartilage-like structures were homogenized and extracted for 24 hr in 1.0 M NaCl containing protease inhibitors. After further homogenization and centrifugation at 30,000 × g for 1 hr, the supernate was dialyzed against 0.1 M acetic acid and lyophilized. Samples of the labeled collagens were dissolved in buffer containing SDS, electrophoresed under reduced conditions in a 6% polyacrylamide gel (PAGE), and analyzed by autofluorography.

Genomic DNA Analysis. Genomic DNA was isolated from cultured fibroblasts as described (21). Ten micrograms of DNA was digested with *Eco*RI, fractionated by electrophoresis overnight on an 0.8% agarose gel, transferred to Hybond-N membrane (Amersham), and hybridized against [^{32}P]dATP (800 μCi/mmol; 1 Ci = 37 GBq) nick-translated COL2A1 cDNA probes (HC-21 and HC-22) spanning the full-length type II collagen message (22), as described elsewhere (21).

PCR Amplification. Genomic PCR amplifications were as described with denaturation at 94°C for 1.5 min, annealing at 55°C for 2.5 min, and extension at 72°C for 3.5 min (12). cDNA amplification of primary overlapping fragments was as described with denaturation at 94°C for 1.5 min, annealing at 55°C for 2.5 min, and extension at 72°C for 2 min (23). Amplification of cDNA subfragments in the single-strand conformation polymorphism (SSCP) analysis was as above, except for extension at 72°C for 0.5 min (24). Oligonucleotide primers used in detecting the mutation are as listed: 5′-TCCTTTAGGACCAGTCACTCCAGTAGGACC-3′, exon 38; 5′-GGGCCCAAAGGCGACAGG-3′, exon 32; 5′-TCCACCATCCTTTCCAGGGGC-3′, exon 33; 5′-GCCCAGGGCCTCCAGGGTCCC-3′, exon 31; 5′-CCCAGCAGTCCAGCAGCC-3′, intervening sequence *33*.

RNA/cDNA Analysis. Total cellular RNA was extracted from the tissue-like, chondrocyte aggregate structures formed in agarose culture. Seven- to ten-day structures (20 mg) were lysed in guanidine isothiocyanate, and the RNA was isolated and purified by centrifugation on a $CsCl_2$ gradient (21). Twenty micrograms was electrophoresed on a 2.2 M formaldehyde/0.8% agarose gel, transferred to Hybond-N membrane (Amersham), and hybridized to a [^{32}P]dATP (800 μCi/mmol) nick-translated COL2A1 cDNA probe (HC-22).

Double-stranded cDNA was synthesized after priming with random hexamers (dN_6), according to manufacturer's recommendations (Amersham). Three overlapping cDNA fragments (exon 1–22, 22–38, and 38–50) spanning the translated domain of *COL2A1* were PCR amplified. These primary amplification products were used for amplification of [^{32}P]dATP internally labeled subfragments for SSCP analysis. For SSCP analysis, 200- to 400-base-pair (bp) fragments were PCR amplified from the primary amplification cDNAs and internally labeled with the addition of 10 μCi of [^{32}P]dATP (800 Ci/mmol) per 100 μl of PCR reaction volume. One microliter of the resulting products was denatured and electrophoresed on a 4% polyacrylamide/[with or without 10% (vol/vol) glycerol] nondenaturing gel at a constant 30 W and autoradiographed overnight at −70°C.

Cloning and Sequencing. Direct sequencing of cDNA and genomic PCR amplification products were done as described (25). cDNA and genomic PCR amplification products were cloned into the *Sma* I site of pUC18 and sequenced by the dideoxynucleotide chain-termination method.

RESULTS

Tissue Studies. The costochondral cartilage histology was typical of hypochondrogenesis. It showed a poorly organized growth plate, unevenly distributed epiphyseal chondrocytes that occupied prominent lacunae, sparse matrix, and extensive fibrous tissue associated with cartilage vascular canals (data not shown). TEM revealed distended rough endoplasmic reticulum (rER) in most epiphyseal chondrocytes and irregular thickening of collagen fibrils (Fig. 1 *C* and *F*) compared with normal controls.

Agarose Culture. The hypochondrogenesis chondrocytes behaved similarly to control chondrocytes in culture. They grew abundantly in monolayer culture. When suspended over agarose, most cells formed a single aggregate per culture within the first 24 hr. The aggregate structures resembled cartilage tissue after 2–3 days, and by 7–10 days they appeared similar to intact cartilage from the infant. Light microscopy showed a decrease in the relative amount of matrix (data not shown), which was confirmed by low-magnification TEM (Fig. 1*A*). The greatest resemblance between the intact cartilage and the culture-derived structures was seen at higher magnification. In both instances the rER was distended with finely granular material in most chondrocytes (Fig. 1*D*), and the collagen fibrils were thick and unevenly distributed (Fig. 1*G*). Although the extent of these changes varied slightly, they were found in almost all fields of several structures examined. The ratio of collagen fibrils to proteoglycan granules appeared reduced in the hypochondrogenesis cultures compared with the controls, but this was not quantitated. The morphology of the control aggregate structures at 7–10 days was essentially that of normal cartilage, as shown for comparison in Fig. 1 *B*, *E*, and *H*.

Collagen Biosynthetic Analysis. PAGE of labeled collagens from control and hypochondrogenesis culture-derived structures is shown in Fig. 2*A* (lanes 1–3). The electrophoretic pattern exhibited by the latter was abnormal. At the α1 position were two bands of equal intensity: one with normal mobility and another with slightly slower mobility. Most label was found in other slower migrating bands, which were not identified but had mobilities expected for proα1(II) chain and incompletely processed proα1(II) chains. The position of these chains was estimated from an experiment in which control cells were or were not treated with ascorbic acid [see PAGE of collagens in Fig. 2*A* (lanes 4 and 5)].

Analysis of Mutation. Southern blot analysis of *Eco*RI digests of genomic DNA from the patient showed fragments of expected size that did not differ from control DNA (data not shown). Northern blot analysis of RNA isolated from the patient's cultured chondrocytes revealed a single COL2A1

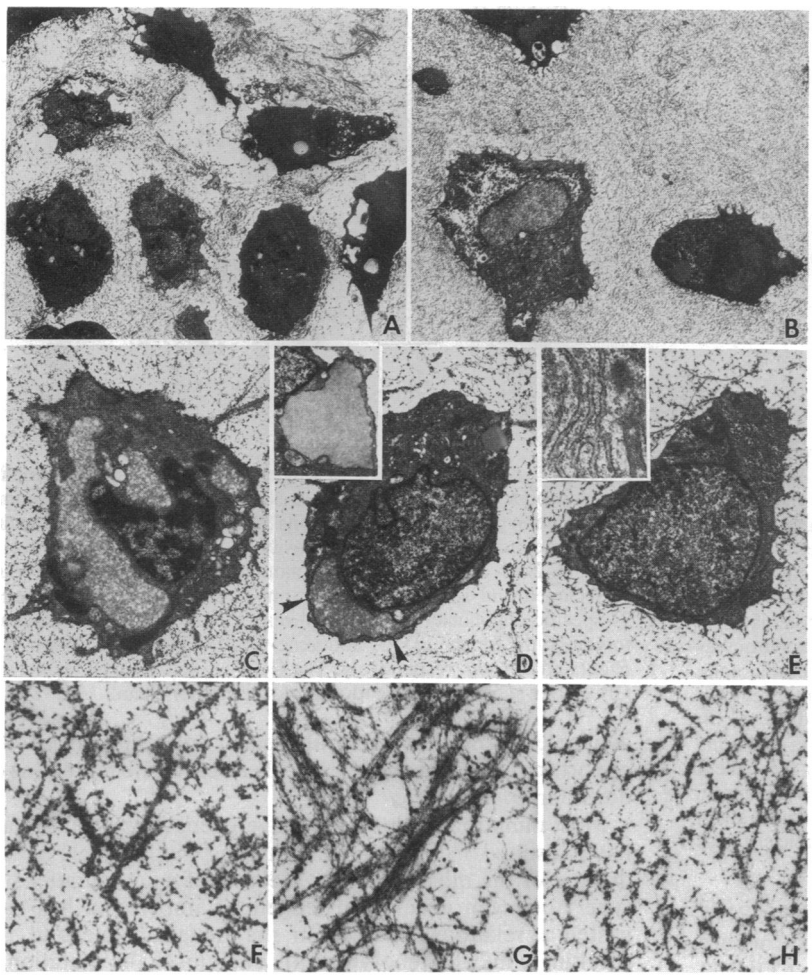

FIG. 1. Composite of electron micrographs. (*A* and *B*) General morphology of the tissue-like structures from 10-day cultures from patient (*A*) and control (*B*) are shown at low magnification; the matrix produced by the hypochondrogenesis chondrocytes in *A* was reduced in amount and disorganized compared with that produced by control chondrocytes in *B*. (*C–E*) Cellular structure of the patient's chondrocytes cultured for 10 days (*D*) closely resembled that of cells from the patient's intact femoral cartilage (*C*) with distended rER (arrows) and differed from the control chondrocytes cultured for 10 days (*E*), which lacked distended rER. (*Insets*) rER of *D* and *E*. (*F–H*) Matrix structure. Most notable was the thickened collagen fibrils seen in 10-day cultures (*G*) and the patient's intact femoral cartilage (*F*), which was absent in 10-day cultures of control chondrocytes (*H*). All cells were cultured in the presence of ascorbic acid. [*A* and *B*, ×500; *C–E*, ×1380 (*Insets*, ×5500); *F–H*, ×5500.]

mRNA transcript ≈5.0 kilobases (kb) in size, which did not differ from control transcripts (Fig. 2*B*).

SSCP analysis of PCR-amplified subfragments from the three overlapping COL2A1 cDNAs suggested an allelic heterozygosity in the region spanning exons 31–33 and 32–38 (Fig. 3). Direct sequencing of this region revealed a heterozygous G → A transition in exon 33 resulting in a Gly → Ser substitution at residue 574 of the proα1(II) triple-helical domain (Fig. 4 *A Left*). Subsequently, a 2.5-kb fragment spanning exons 32–38 was amplified from patient's genomic DNA. Direct sequencing of exon 33 revealed the same G/A heterozygosity (Fig. 4 *B Left*). The G/A heterozygosity was confirmed on both the cDNA and genomic level by cloning and sequencing of each allelic product (Fig. 4 *A* and *B Right*). The mutation was not found in either parent's genomic DNA, as determined by SSCP analysis of exon 33 (data not shown).

DISCUSSION

We report an approach to investigating mutations that disrupt chondrogenesis and cause certain human chondrodysplasias. This approach was used to detect a *COL2A1* mutation in human hypochondrogenesis. The mutation was a G → A transition in codon 574 that caused a Gly → Ser substitution in the middle of the proα1(II) triple-helical domain. In this study the consequences of a *COL2A1* mutation have been examined *in vitro*.

Our strategy used cultured differentiated chondrocytes as a source of materials for analysis. When grown under conditions that promote attachment, mammalian chondrocytes typically assume a fibroblastic appearance and lose their chondrocytic pattern of gene expression—i.e., the predominant collagen synthesized switches from type II to type I collagen (18). However, such "dedifferentiated" cells proliferate extensively. When attachment is prevented by culture over a substance such as agarose, proliferation ceases, but the differentiated phenotype is restored (18–20). We exploited this phenotypic plasticity to generate sufficient numbers of appropriately differentiated cells for study. Although some lines of human epiphyseal chondrocytes do not redifferentiate under the conditions used in these experiments, most do in our experience. Moreover, while most of our studies have been done on cells passaged in monolayer culture fewer than five times, we have observed redifferentiation after as many as nine passages.

The conditions used here were similar to a previously published method (19), except that the cells were suspended over rather than in agarose. This was an important difference because it promoted cell aggregation and the formation of tissue-like structures that could be analyzed by established techniques. These structures strongly resembled cartilage grossly, histologically, and ultrastructurally. Similarly, electrophoretic analysis of labeled collagens demonstrated the synthesis of cartilage collagens—primarily type II collagen. Thus, the culture scheme provided a valid experimental model of chondrogenesis. It offered the opportunity to monitor the synthesis of matrix proteins and the elaboration of matrix. By observing the formation of the cartilage-like structures over time, it provided an estimate of the overall effectiveness of chondrogenesis and, with regard to chondrodysplasias, insight into the ability of chondrocytes to generate cartilage templates.

With one exception, previous reports of *COL2A1* mutations have resulted from analysis of genomic DNA. While this approach obviates the need for cartilage tissue or chondrocytes, it is time- and labor-intensive. Our strategy of

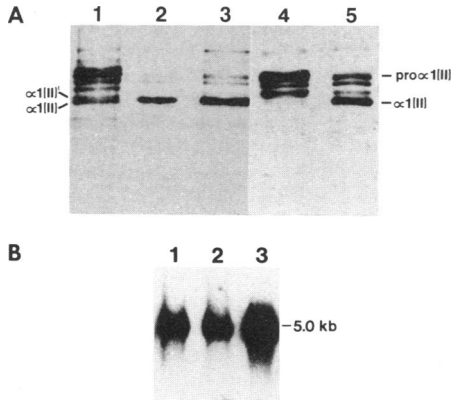

FIG. 2. (*A*) PAGE of [^{3}H]proline-labeled collagen preparations derived from 10-day chondrocyte cultures. Ascorbic acid was present in all cultures except for that of lane 4, and PAGE was done under reduced conditions. Lanes: 1, patient, showing two bands of very similar mobility in the α1 position and several prominent bands of slower mobility; 2 and 3, 7-day-old control chondrocyte culture (two concentrations; slightly less collagen was added to lane 3 than to lane 1). A single band with the mobility of α1(II) chain predominated in controls. Lanes 4 and 5, experiment in which control chondrocytes were cultured with or without ascorbic acid; 5, with ascorbic acid, bands with mobilities for α1(II) chain and, to a lesser extent, predicted for proα1(II) chain and processing intermediates were detected; 4, without ascorbic acid most label was detected in the slower migrating bands of comparable mobility to those predominating in the patient (lane 1), suggesting impaired conversion of proα1(II) to α1(II) chains in both instances. (*B*) Northern (RNA) blot analysis of RNA from patient (lane 1) shows expected 5.0-kb size of COL2A1 mRNA transcripts as also found in two controls (lanes 2 and 3).

synthesizing cDNA from culture-derived total cellular RNA and analyzing it by PCR-based methods was straightforward and much more efficient. The full-length human COL2A1 cDNA is only ≈5 kb compared with the 38-kb human *COL2A1* gene (26). The cDNA was readily analyzed by PCR amplification of three overlapping fragments, SSCP analysis, and direct sequencing. Chan and Cole (27) have recently identified a *COL2A1* mutation from analysis of cDNA derived from "illegitimate" COL2A1 transcripts produced by fibroblasts and lymphoblasts. This approach has great potential for detecting mutations when the affected tissue is not

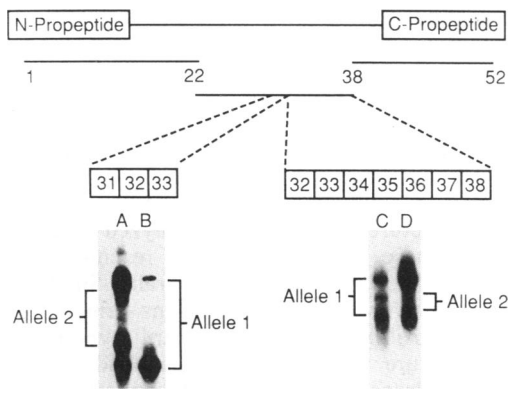

FIG. 3. Schematic representation of primary (exons 1–22, 22–38, 38–50) and secondary cDNA amplification products and SSCP analysis of *COL2A1* exons 31–38. Lanes: A and B, SSCP analysis of secondary PCR amplification products containing exons 31–33 from patient and control primary cDNA amplification products, respectively; C and D, SSCP analysis of cDNA secondary products containing exons 32–38 from patient and control amplifications, respectively. Alleles 1 (upper and lower bands) and 2 (middle bands) represent the relative mobilities of the Watson–Crick strands of DNA derived from the normal and mutant alleles, respectively.

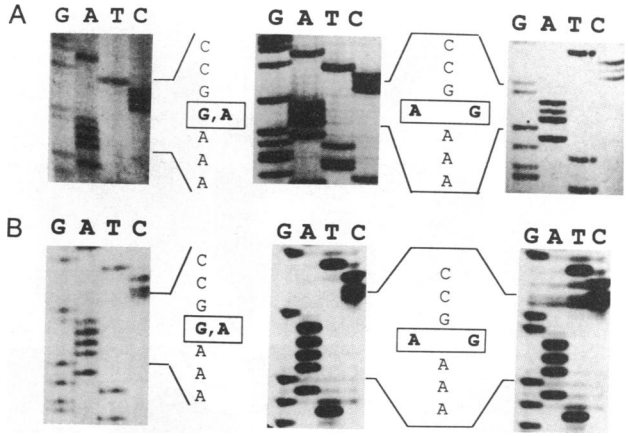

FIG. 4. Direct sequencing of single-strand patient cDNA amplification products containing exons 22–38 (*A Left*) and patient genomic amplification products spanning exons 32–38 (*B Left*). This sequencing reveals a consistent G/A heterozygosity on the RNA and gene level in exon 33. Each allelic cDNA and genomic PCR amplification product was cloned and sequenced with exon 31 and intervening sequence *33*-specific oligonucleotide primers, respectively. The sequencing autoradiograms for the cDNA and gene-specific alleles are shown in *A Right* and *B Right*, respectively. The GGC → AGC change results in a Gly → Ser substitution at codon 574 of proα1(II) chain.

available. However, their approach does not afford the opportunity to study the biological effects of the mutations as does our approach.

Most mutations of human collagen genes reported to date have resulted in glycine substitutions in the triple-helical domain of the molecules (28). Review of such mutations of type I collagen genes in osteogenesis imperfecta has suggested a relationship between the mutation site and the resulting clinical phenotype (29). The closer the mutation is to the carboxyl terminus of the triple helix, the more severe the phenotype. Because the helix folds in a carboxyl-to-amino direction, it has been postulated that glycine substitutions near the carboxyl terminus disrupt folding more than those at the amino terminus in a graded fashion. However, as exceptions to this rule increase, it is becoming evident that additional factors must also contribute to the severity of the resulting clinical phenotype (30–32). This conclusion is underscored by the three Gly → Ser substitutions now identified in COL2A1 (13, 27). A mild SED phenotype resulted from a mutation at amino acid residue 997 (exon 48), whereas hypochondrogenesis, which lies near the severe end of the SED clinical phenotypic spectrum, resulted from mutations at residue 943 (exon 46) and residue 574 (exon 33, this case).

Distention of the rER was seen in hypochondrogenesis chondrocytes but was not found in control chondrocytes cultured under the same conditions. Similar distention was seen in the intact epiphyseal cartilage from this case. Because such distention is found commonly in SED cartilage in general, it has been proposed that this distention reflects defective transport from the endoplasmic reticulum of type II procollagen molecules containing mutant proα chains. Indeed, granular material in rER was stained with antibodies to type II collagen in the other case of hypochondrogenesis in which a *COL2A1* mutation was demonstrated (8). Presumably, the normal transport process is disturbed by abnormal folding or other abnormalities of procollagen molecule caused by the mutation. That the material present in rER in this case was abnormal type II procollagen is supported by the morphologic findings. Microscopy showed that the relative amount of cartilage matrix was less in the cartilage-like structures from hypochondrogenesis cultures than in control cultures. In addition, an apparent reduction of collagen fibrils relative to proteoglycan granules was observed ultrastructurally.

The electrophoretic studies further support defective conversion of type II procollagen to collagen. Compared to control cultures in which almost all the 24-hr proline label migrated in the α1(II) position by PAGE, a much smaller proportion of the label was found in this position in cultures from the case. The bulk of the label was found in bands having the mobility of proα1(II) chain and processing intermediates. We were unable to obtain sufficient culture-derived collagen from this case to confirm this or exclude that these bands were derived from other procollagen types by two-dimensional gel electrophoresis of cyanogen bromide peptides. However, strong indirect evidence was provided by experiments in which electrophoretic bands of comparable mobility were observed when control chondrocytes were cultured in the absence of ascorbic acid, which is known to disrupt post-translational processing of procollagen chains.

Glycine substitutions in α1(I) chains that cause osteogenesis imperfecta are usually associated with overmodification of the majority the α1(I) chains detected electrophoretically (33–35). This situation reflects the stoichiometry of the molecule—i.e., at least one mutant chain is present in three-fourths of procollagen molecules. The stoichiometry of type II procollagen predicts that seven-eighths of the molecules would contain at least one mutant α1(II) for heterozygous mutations of *COL2A1*. Such overmodification has been demonstrated in several cases of moderate-to-severe SED (11); however, this overmodification was not seen in this case. A putative overmodified α1(II) chain with slightly slower electrophoretic mobility was seen, but it was essentially the same intensity as the normally migrating α1(II) chain. If it were an overmodified α1(II) chain, the finding implies either that fewer of the mutant proα(II) chains were incorporated into procollagen molecules than predicted or that many overmodified proα1(II) molecules were not processed to collagen molecules. The latter possibility seems much more likely, given the rER distention and electrophoretic observations.

Structurally abnormal collagen fibrils were found in both intact cartilage and culture-derived structures from our case. Because we biochemically analyzed collagens from the entire cartilage-like structures without distinguishing between their intracellular and extracellular origin, the extent to which collagen molecules containing the mutant α chains were incorporated into these fibrils is not known. The abnormal fibrils could, therefore, reflect the direct effects of abnormal type II collagen molecules on fibril assembly, the effects of a diminished amount of normal type II collagen molecules relative to types IX and XI collagen molecules on this process, or some combination.

In summary, our findings indicate that a heterozygous G→A transition in *COL2A1* caused a glycine substitution in the middle of the type II collagen triple helix. This substitution appears to have interfered with the conversion of type II procollagen to collagen, impaired the intracellular transport and secretion of the molecule, and disrupted the extracellular assembly of cartilage collagen fibrils. The net effect was apparently a quantitatively deficient and qualitatively defective cartilage matrix that functioned poorly as a template for embryonic bone formation and subsequent linear bone growth. The experimental approach used in this investigation differed from previous approaches and provided the means not only to detect the mutation but also to examine its effects on type II collagen synthesis and on chondrogenesis, in general. The approach should be applicable to the investigation of a wide variety of defects of chondrogenesis.

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