The Fate of Cartilage Oligomeric Matrix Protein Is Determined by the Cell Type in the Case of a Novel Mutation in Pseudoachondroplasia

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We have identified a novel missense mutation in a pseudoachondroplasia (PSACH) patient in one of the type III repeats of cartilage oligomeric matrix protein (COMP). Enlarged lamellar rough endoplasmic reticulum vesicles were shown to contain accumulated COMP along with type IX collagen, a cartilage-specific component. COMP was secreted and assembled normally into the extracellular matrix of tendon, demonstrating that the accumulation of COMP in chondrocytes was a cell-specific phenomenon. We believe that the intracellular storage of COMP causes a nonspecific aggregation of cartilage-specific molecules and results in a cartilage matrix deficient in required structural components leading to impaired cartilage growth and maintenance. These data support a common pathogenetic mechanism behind two clinically related chondrodysplasias, PSACH and multiple epiphyseal dysplasia.

Mutations in COMP, cartilage oligomeric matrix protein, have been associated with a bone dysplasia family that includes pseudoachondrodysplasia (PSACH) and multiple epiphyseal dysplasia (MED). PSACH and MED (EDM1) were localized to chromosome 19p13.1, the region that contains the gene for COMP (1–4), and specific base substitutions, deletions, and duplications have been subsequently identified (5, 6). Both PSACH and MED are inherited as dominant disorders.

The clinical features of the two diseases are similar and can range from mild to severe forms (7). PSACH is characterized by a disproportionate short stature and joint laxity with a waddling gait that appears with the onset of walking. MED patients generally present with mild short stature and hip pain later in childhood. The radiological abnormalities of MED are restricted to the epiphyses whereas there is additional involvement of metaphyses and the spinal column in patients with PSACH. Patients with both disorders manifest symptoms of precocious osteoarthritis. The diseases appear to be allelic variants resulting from different mutations in the same gene.

Another extracellular matrix component of cartilage, type IX collagen, has also been implicated in MED. This form of MED (EDM2) affects primarily the growth centers of the knees and has been mapped to chromosome 1p32 (8) in a family that had previously shown no linkage to COMP (chromosome 19p13). COL9A2 was also mapped to this locus (9). The major complaint of this MED family was early onset osteoarthritis, especially in the knees. Only a few individuals exhibited mild short stature, distinguishing this family from others with MED. Recently, a mutation in the α2 chain of type IX collagen was identified in this large family (10). It is not clear how mutations in these two genes result in a similar phenotype.

COMP is the fifth member of the thrombospondin (TSP) family of extracellular matrix glycoproteins (3). It was originally discovered in cartilage extracts and has been immunolocalized to developing as well as mature cartilage (11, 12). COMP has also been found within and around tendon bundles (13). Rotary shadow imaging shows the molecule to be composed of 5 flexible arms with a large globular domain at the end of each arm (14). Assembly of the oligomer requires the formation of an α-helical coiled-coil domain that is later stabilized by interchain disulfide bonds (15). The region between the assembly domain and the terminal knob consists of epidermal growth factor-like and TSP type III repeats (3). It is within these type III repeats where mutations that cause PSACH and MED are clustered (5, 6, 16). The type III repeats of TSP1 have previously been shown to bind calcium (17–19). Therefore, it is presumed that the homologous type III repeats of COMP also bind calcium.

Ultrastructural analysis of chondrocytes from patients with PSACH and MED show distinctive and enormous rough endoplasmic reticulum (rER) vesicles (20–22). The material within the vesicles is composed of alternating electron-lucent and electron-dense layers that have a unique lamellar appearance. The role of these large storage vesicles in the pathophysiology of the dysplasias is not clear. A possible mechanism for impaired cartilage function could be related to the intracellular accumulation of mutant molecules as suggested by the enlarged rER.
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vesicles. This could lead to a deficiency of extracellular matrix components or possibly adversely affect chondrocyte metabolism or proliferation.

The fate of a COMP molecule containing a mutation has not been established. If both the wild-type and mutant alleles are equally transcribed and translated, the likelihood of a pentameric COMP molecule containing one or more mutant chains is 97%. Based upon studies of TSP1 (23), pentamer formation is thought to occur intracellularly, but whether mutant chains can be incorporated into pentameric COMP and what effect mutant chains may have on the structure of COMP are not known. Also unknown is whether mutant COMP molecules are secreted into the extracellular matrix or retained in the unusual rER vesicles characteristic of these diseases. In the assembly of a COMP pentamer, different numbers of mutant chains could be incorporated, assuming this process is random, and different populations of molecules may be sorted by the cell in a similar or different manner.

Evidence presented here demonstrates that a mutation in COMP results in intracellular accumulation of the COMP pentamer specifically within the unusual rER vesicles in PSACH chondrocytes. Moreover, we show that this accumulation does not occur in an adjoining tendon, suggesting that the intracellular fate of mutant COMP is determined by the chondrocyte environment and not simply by structural effects of the mutation on COMP. Finally, we demonstrate that type IX collagen, a cartilage-specific molecule, is also accumulated within the rER vesicles, suggesting a pathogenetic link between PSACH and MED.

EXPERIMENTAL PROCEDURES

Patient Samples—The patient was a 10-year-old male with typical features of PSACH (disproportionate short stature, joint laxity, and abnormal radiological findings) consistent with the diagnosis. A cartilage sample that included some of the adjacent tendon was removed from iliac crest following informed consent. Chondrocytes were isolated following collagenase digestion of cartilage: 3 mg/ml collagenase D (Boehringer) in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 5% fetal calf serum overnight at 37 °C. The isolated cells were cultured for up to 8 passages resulting in a “dedifferentiated” phenotype.

Mutation Analysis—Genomic DNA was isolated from chondrocytes from the PSACH patient and 50 unrelated normal individuals. 30 rounds of PCR amplification were carried out at an annealing temperature of 60 °C using primers for exon 17B. The forward primer (5'-CTGGAGAAGCTTGTCTGTGG-3') recognized a sequence in the 5'-flanking intron, and the reverse primer (5'-CTAACCGCGCCGAGGAG-3') was within exon 17B. Direct sequence analysis of the PCR fragment was carried out using an ABI DNA Sequencer with end-labeled primers as described above. As the identified mutation eliminated an AvaI cleavage site, the patient and the 50 normal PCR fragments were digested with AvaI.

Preparation of Antibodies—Polyclonal (pAb 3593) and monoclonal antibodies (mAb 5-1) to COMP were produced using material purified from the medium of cultured human ligament cells. COMP was first fractionated by DEAE-chromatography (Waters HPLC) and then concentrated using Mono Q chromatography (Pharmacia) to remove lower molecular weight contaminants and then eluted from the medium of cultured human ligament cells. COMP was first fractionated by DEAE-chromatography (Waters HPLC) and then concentrated using Mono Q chromatography (Pharmacia). The antibody to type II collagen was produced in goat and is commercially available (Southern Biotech Inc.). Fractions containing COMP were sieved (Superose 6, Pharmacia) to remove lower molecular weight contaminants and then eluted from the medium of cultured human ligament cells. COMP was first fractionated by DEAE-chromatography (Waters HPLC) and then concentrated using Mono Q chromatography (Pharmacia). The antibody to type II collagen was produced in goat and is commercially available (Southern Biotech catalog no. 1320-01).

Immunocytochemistry—10 μm of frozen cartilage and tendon sections were acetone-fixed and stained with antibodies to COMP, types II and IX collagens, and with and without chondroitinase pretreatment (0.2 unit/ml in 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM sodium chloride, 1 h at room temperature). Cultured chondrocytes from the

FIG. 1. Identification of a novel mutation in COMP in a PSACH patient. A model of the COMP pentamer shows prominent features of the molecules; the coiled-coil domain at the amino terminus, the flexible arms composed of a linear array of epidermal growth factor-like domains and TSP type III repeats that terminate in a globular domain at the carboxyl terminus. The mutation is located in the 6th type III repeat and results in the substitution of asparagine for aspartic acid as identified by the arrow underneath the mutant amino acid sequence.

PSACH patient sample were fixed and stained with antibodies to COMP as above.

Ultrastuctural Analysis—The ultrastructure of the cartilage was examined following fixation in cacodylate-buffered 1.5% paraformaldehyde, 1.5% glutaraldehyde containing 0.1% tannic acid, followed by postfixation in osmium tetroxide, dehydration in ethanol and propylene oxide, and embedded in Spurr epoxy as described previously (25). Additionally, some cartilage was fixed for 30 min on ice in cacodylate-buffered 0.1% glutaraldehyde, 4% paraformaldehyde, rinsed in buffer, and immersed in increasing concentrations of ethanol from 30% to 90% at increasingly lower temperatures from 0 °C to −20 °C. Samples were infiltrated with LR White resin at −20 °C, then slowly warmed to 60 °C, and polymerized. Sections cut from these blocks were labeled using antibodies to COMP (pAb 3593) and type IX collagen (pAb 9264) via the surface labeling procedure described previously (25).

Microsome Isolation and Protein Labeling—Chondrocytes were isolated from cartilage following overnight collagenase digestion (see above for details). The cells were homogenized and fractionated as described previously (26). The pellet debris fraction was resuspended in 5 mM Hepes, pH 6.8, containing 250 mM sucrose, 25 mM KCl, and 5 mM MgCl₂. An aliquot for electron microscopy was sedimented, and the resulting pellet was analyzed. The remainder was resuspended in SDS-PAGE sample buffer (12.5 mM Tris-HCl buffer, pH 6.8, containing 2.0% glycerol, 0.01% bromophenol blue, in the presence or absence of 2.5 mg/ml dithiothreitol) and boiled. Following electrophoresis the gel was transferred to a polyvinylidene difluoride membrane in 10 mM CAPS buffer, pH 11, containing 10% methanol. The membrane was incubated with antibodies to COMP (pAb 3593) and type IX collagen (pAb 9264). Horseradish peroxidase-conjugated secondary antibodies were detected by chemiluminescence (Pierce Super Signal).

RESULTS

Identification of a New Mutation in the COMP Gene—Direct sequencing of the exon 17B PCR fragment from patient genomic DNA revealed a single base substitution that changed an aspartic acid (GAC) to an asparagine (AAC) at position 346 (Fig. 1). This mutation eliminated an AvaI restriction enzyme cleavage site. The PSACH DNA and DNA from 50 laboratory controls were amplified and digested with AvaI. The control DNA samples showed two fragments while the PSACH DNA showed a mixture of the cleaved products and a larger band representing the uncleaved fragment (data not shown). The mutation affected a conserved residue in the sixth TSP type III repeat.

Immunofluorescence of COMP and Type IX Collagen in PSACH Cartilage and Tendon—Normally, COMP is found in the extracellular matrix of cartilage, and the matrix does not require predigestion of proteoglycans for visualization of COMP by immunofluorescence (data now shown). In contrast, type IX collagen requires predigestion of proteoglycans to visu-
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The antibody staining pattern of COMP in tendon differed from that of cartilage. While there was almost complete inclusion of COMP in chondrocytes, there was pronounced extracellular staining of tendon (Fig. 2d). Antibodies to type IX collagen, a collagen not expressed by tendon fibroblasts, did not label tendon above background (Fig. 2e). Cultured chondrocytes were also stained with COMP antibodies. In these dedifferentiated cells, COMP was incorporated into the extracellular matrix with no indication of intracellular accumulation (data not shown), consistent with what was found in tendon.

Ultrastuctural Localization of COMP and Type IX Collagen in PSACH—The ultrastructural analysis of cartilage and cellular fractions from the PSACH patient showed the intracellular vesicles characteristic of PSACH/MED chondrocytes (Fig. 3a). The material within these enlarged rER vesicles was organized into alternating layers of electron-dense and electron-lucent material. In addition to the resulting lamellar pattern, there were also vesicles that appeared to have less organization of the intracellular material. In these cases, the plane of section may obscure the architecture of the stored components within the rER.

Immunoelectron microscopy localized COMP to the vesicles. While there was some matrix labeling with the antibody, the enlarged rER vesicles were heavily labeled with gold particles (Fig. 3b). The darker, more electron-dense rings within the lamellar-like structures were preferentially labeled over the lighter layers. Type IX collagen was also localized to the rER vesicles, also preferentially to the darker rings (Fig. 3c).

Biochemical Analysis of the rER Vesicles of PSACH Chondrocytes—Primary chondrocytes were isolated and fractionated by centrifugation, and the contents were analyzed by electron microscopy (Fig. 4A) and Western blot analysis (Fig. 4B). The pellet debris fraction was shown to contain vesicles that displayed the lamellar pattern seen in intact cells. This fraction was separated by SDS-PAGE and transferred to a polyvinylidene difluoride transfer membrane (Bio-Rad). A single band at approximately 100–110 kDa, the molecular mass for a COMP subunit, was recognized by COMP antibodies under reducing conditions. A broad, high molecular weight band representing the oligomeric form of COMP was present in the non-reduced sample. A band at the approximate position of the type IX collagen trimer was recognized by type IX antibodies without reduction.

DISCUSSION

We have described a novel mutation of COMP in a patient with PSACH, resulting in the substitution of asparagine for aspartic acid in one of the TSP type III repeats. In addition, to address questions of the pathogenesis of PSACH and MED, we have examined the unusual contents of chondrocytes from this patient, using both immunohistochemical and biochemical/cell fractionation techniques.

Several significant observations were made. First, we demonstrated that COMP is primarily accumulated inside chondrocytes in PSACH and showed that COMP is specifically localized to the unusual fingerprint patterns found in the rER vesicles which are characteristic for PSACH. These data suggest that the normal extracellular fate of wild-type COMP molecules is altered in a profound dominant negative manner by mutant COMP molecules in PSACH cartilage. Second, we showed that this effect of mutant COMP molecules was cartilage-specific, since the presence of COMP in the extracellular matrix of PSACH tendon was apparently normal. Similarly, we found that COMP molecules are secreted into the medium (and not retained intracellularly) by dedifferentiated PSACH chondrocytes (data not shown). Third, we found that type IX collagen, a cartilage-specific collagen, was also accumulated in PSACH chondrocytes, in contrast to type II collagen, another cartilage-specific collagen, which was secreted and incorporated into cartilage extracellular matrix. These data suggest that the pathogenesis of PSACH, and possibly also MED, involves the interaction(s) of the mutant COMP gene products with specific cartilage components, principally type IX collagen, and is not due solely to the effect of mutant molecules on the formation and secretion of COMP pentamers. If the latter were the case, then COMP molecules should have been retained inside the PSACH tendon fibroblasts and dedifferentiated chondrocytes.

By immunoblotting, we have demonstrated that COMP assembles into its native pentameric form. However, a very broad, high molecular weight band was seen in the unreduced lane (Fig. 4b) which suggests two possible aberrant events. The mutation may result in abnormal glycosylation. However, this
seems unlikely since the reduced chain appears as a single sharp band. Alternatively, there may be a mixed population of disulfide-bonded multimers. We speculate that this mutation may prevent formation of the normal disulfide bond that utilizes the cysteine residue just carboxyl-terminal to the substituted asparagine, creating free sulfhydryl groups. Free sulfhydryls may interact in an abnormal manner with other cysteines forming different molecular species, depending on how many mutant chains are present in the pentamers. This could lead to the broad band seen in the unreduced sample by SDS-PAGE.

Abnormally folded COMP molecules may be retained by the rER chaperones that associate with large oligomeric molecules (27), leading to accumulations of molecules in the ER.

The accumulation of type IX collagen along with COMP in the PSACH chondrocytes raises questions about possible interactions between these molecules. In normal cartilage matrix, these two molecules may interact in a biologically functional manner. The presence of aggrecan in the vesicles of PSACH chondrocytes has been documented (28, 29). However, specific extracellular interactions, such as a COMP-type IX collagen interaction or a COMP-type IX collagen-aggrecan association, have not been described. Immunolocalization of COMP antibodies in normal cartilage has failed to colocalize the protein to type IX collagen-containing fibrils (data not shown). Therefore, we propose that the mutation in COMP causes a nonspecific aggregation of COMP, type IX collagen, aggrecan, and possibly other chondrocyte-specific molecules present simultaneously in the ER. Previous work has demonstrated that the secretory pathways in chondrocytes for type II collagen and aggrecan are different (30). It is possible that COMP and type IX collagen share a common pathway with aggrecan distinct from that of type II collagen. This would explain the normal secretion and extracellular matrix deposition of type II collagen that we see in the cartilage from the PSACH patient.

Mutations in type IX collagen have been introduced into two mouse strains to obtain further insight into its role in cartilage (31, 32). A transgenic mouse strain was established with a central deletion of the \( \alpha_1(IX) \) collagen chain that resulted in mild dwarfism associated with osteoarthritis (32). A second mouse strain was created that lacked the \( \alpha_1(IX) \) collagen chain (31). These mice developed severe osteoarthritis, similar to the transgenic mice, and to the human diseases associated with type IX collagen and COMP.

Taken together, our observations allow us to conclude that the intracellular storage of COMP results in the PSACH phenotype through two potential mechanisms. First, a reduction in the amount of COMP in the extracellular space may lead to a structurally compromised cartilage matrix and impairment of cartilage growth. Second, abnormal retention of COMP inside the chondrocyte may affect normal cellular metabolism. The pathogenetic relationship between the PSACH and MED is
intriguing because of the presence of type IX in PSACH rER vesicles. To more fully appreciate the underlying similarities, it will be important to know what the fate of type IX collagen is in the MED family with the mutation in COL9A2. If the amount of type IX collagen in the matrix is reduced, independent of its effects on COMP, it is likely that these diseases may result primarily from a reduction of structural components of the extracellular matrix (principally of type IX collagen), similar to other disorders resulting from mutations in type II collagen (33).

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