Physiological and Pathological Secretion of Cartilage Oligomeric Matrix Protein by Cells in Culture*  

(Received for publication, May 14, 1998, and in revised form, July 9, 1998)  

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Abnormalities in cartilage oligomeric matrix protein (COMP), a pentameric structural protein of the cartilage extracellular matrix, have been identified in pseudo-achondroplasia and multiple epiphyseal dysplasia, two human autosomal dominant osteochondrodysplasias. However, the function of the protein remains unknown. With the goal of establishing a model to study the mechanisms by which COMP mutations cause disease, we have analyzed synthesis and secretion of COMP in cultured chondrocytes, tendon, and ligament cells. Patient cells expressed mutant and normal RNA and secreted COMP at levels similar to controls, suggesting that abnormal pentamers are likely to be found in the extracellular matrix. Inclusions within patient cartilage stained with anti-COMP antibodies, and cultured cells presented similar inclusions, indicating that presumably abnormal COMP pentamers are less efficiently secreted than normal molecules. We conclude that the COMP disorders are likely to result from a combination of a decreased amount of COMP in the matrix and a dominant negative effect due to the presence of abnormal pentamers in cartilage.

Cartilage oligomeric matrix protein (COMP) is a 524-kDa homopentameric extracellular matrix glycoprotein, which belongs to the thrombospondin family of proteins (1–4). Each monomer is composed of an amino-terminal cysteine-rich domain, four EGF-like domains, eight calmodulin-like repeats, and a C-terminal globular domain. The cysteine-rich domain is responsible for assembly of the monomers into pentamers by interchain disulfide bonds (5). The COOH-terminal domain may be involved in binding cells, such as chondrocytes (6), and proteins in the extracellular matrix (7, 8).

COMP has recently been identified as the abnormal protein in two human autosomal dominant skeletal dysplasias, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED) (9, 10). In addition to mutations in COMP, MED can also result from mutations in the gene encoding the protein, multiple epiphyseal dysplasia; MED, multiple epiphyseal dysplasia; PSACH, pseudoachondroplasia; TGB-β, transforming growth factor β; RER, rough endoplasmic reticulum.

* This work was supported in part by Grants HD22567 and AR43139 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: COMP, cartilage oligomeric matrix protein; MED, multiple epiphyseal dysplasia; PSACH, pseudoachondroplasia; TGB-β, transforming growth factor β; RER, rough endoplasmic reticulum.

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EXPERIMENTAL PROCEDURES

Cell Lines and Patients—Cultures were derived from control and patient tissues referred to the International Skeletal Dysplasia Registry at the Cedars-Sinai Research Institute. Primary cell cultures were established from knee ligament, Achilles’ tendon, and proximal and distal femoral cartilage from a 28-week-old control human male fetus (International Skeletal Dysplasia Registry reference number 1096-3722). Cartilage and cell extracts, and cell supernatants, were partially digested with Auto/Zyme solution (Biomeda). The supernatants and cell layers were collected separately and analyzed by polyacrylamide gel electrophoresis, Western blotting, and immunoprecipitation. In the supernatants of the cultured ligament cells, we identified a protein that co-migrated with partially purified COMP from fetal cartilage (Fig. 1, lane 1) and that could be immunoprecipitated with anti-COMP antibodies (Fig. 1, lane 2). Reduction of the immunoprecipitated material yielded a protein that co-migrated with monomeric COMP (Fig. 1, lane 5). In the absence of antibody (Fig. 1, lane 6), this protein was not observed. Comparable results were also obtained with cultured tendon cells and chondrocytes (data not shown). In the immunoprecipitation experiments, a second protein, with a molecular mass of 130–140 kDa after reduction, was also apparent (Fig. 1, lane 5). It is possible that this molecule is monomeric thrombospondin-4, the closest member to COMP in the thrombospondin family of proteins, but we did not test this hypothesis. This protein was not detected in purified cartilage extracts.

We tested the effect of transforming growth factor-β1, a growth factor known to affect bone and cartilage growth and/or differentiation, and that stimulates COMP synthesis by cultured synovial cells (28) on COMP biosynthesis by the cell lines. Treatment with soluble purified TGF-β1 strongly stimulated COMP secretion in cultured ligament (Fig. 1, lanes 7 and 8), tendon (Fig. 1, lanes 10 and 11), and chondrocytes (Fig. 1, lanes 15 and 16). The general metabolism of the cells was enhanced by this treatment, but a few proteins, including COMP, were remarkably more stimulated than others (e.g. Fig. 1, lanes 15 and 16). Ascorbic acid, which is known to enhance collagen secretion (29), did not affect COMP synthesis (data not shown).

For the cultured ligament cells, only the pentameric form of COMP could be detected in the supernatant and the cell layer samples, suggesting that the pentamer is assembled intracellularly (Fig. 2, result b). Immunohistochemical results were also obtained with cultured chondrocytes (Fig. 2e) and tendon cells (data not shown).

RESULTS

COMP Synthesis by Cultured Cells—COMP biosynthesis was examined in cultured cells derived from cartilage and tendon, tissues known to contain COMP. Since joint laxity is a major aspect of the phenotype of PSACH, we also examined cultured ligament cells. All three cell types were grown in monolayer and subjected to steady-state labeling with [35S]cysteine. The supernatants and cell layers were collected separately and analyzed by polyacrylamide gel electrophoresis, Western blotting, and immunoprecipitation. In the supernatants of the cultured ligament cells, we identified a protein that co-migrated with partially purified COMP from fetal cartilage (Fig. 1, lane 1) and that could be immunoprecipitated with anti-COMP antibodies (Fig. 1, lane 2). Reduction of the immunoprecipitated material yielded a protein that co-migrated with monomeric COMP (Fig. 1, lane 5). In the absence of antibody (Fig. 1, lane 6), this protein was not observed. Comparable results were also obtained with cultured tendon cells and chondrocytes (data not shown). In the immunoprecipitation experiments, a second protein, with a molecular mass of 130–140 kDa after reduction, was also apparent (Fig. 1, lane 5). It is possible that this molecule is monomeric thrombospondin-4, the closest member to COMP in the thrombospondin family of proteins, but we did not test this hypothesis. This protein was not detected in purified cartilage extracts.

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Synthesis and Secretion of COMP by PSACH and MED Patient Cells—We examined COMP biosynthesis by cultured cells from PSACH and MED patients. The three patients studied had distinct mutations in one of the calmodulin-like domains of COMP. In addition, for all three patients, direct sequence analysis of reverse transcription-polymerase chain reaction products containing the mutations demonstrated approximately

2 L. M. King and D. H. Cohn, unpublished data.
3 E. Delot, L. M. King, M. D. Briggs, W. R. Wilcox, and D. H. Cohn, submitted for publication.
Secretion of COMP in Control and Patient Cells

**Fig. 1.** Secretion of COMP by control and patient cultured cells. Lanes 1–3, electrophoresis under nonreducing conditions. The COMP pentamer is identified by the arrowhead. Lane 1, supernatant from ^35^S-labeled control fetal ligament cells. Lane 2, immunoprecipitation of the ligament cell supernatant with anti-COMP antibody. Lane 3, Western blot of partially purified COMP from cartilage. Lanes 4–16, electrophoresis under reducing conditions. The COMP monomer is identified by the arrowhead. When applicable, treatment of cells with (+) or without (−) TGF-β is indicated. Molecular mass marker sizes, in kilodaltons, are indicated on the right. Lane 4, Western blot of partially purified COMP from cartilage. Lanes 5 and 6, supernatants from ^35^S-labeled control fetal ligament cells, immunoprecipitated with and without anti-COMP antibody, respectively. Lanes 7 and 8, ligament cell supernatants from cells labeled in the presence and absence of TGF-β, respectively. Lanes 10 and 11, tendon cell supernatants from cells labeled in the absence and presence of TGF-β, respectively. Lanes 9 and 12, supernatants from tendon cells from MED patient R95-204 labeled in the absence and presence of TGF-β, respectively. Lanes 13 and 14, chondrocyte supernatants from PSACH patient R85-160A and MED patient R95-204, respectively.

**Fig. 2.** The COMP pentamers are assembled intracellularly. a, immunoprecipitates derived from the supernatant (S) and cell layer (C) of ^35^S-labeled cultured fetal ligament cells separated by electrophoresis under nonreducing conditions. b, Western blot with anti-COMP antibodies of proteins synthesized by cultured fetal ligament cells. c, Western blot of proteins synthesized by cultured chondrocytes from a control adult (C1) and patients R85-160 (C2) and R91-213A (C3 and S3). The presence (+) or absence (−) of TGF-β in the culture medium is indicated. Molecular mass marker sizes, in kilodaltons, are shown on the right. The arrowhead indicates the COMP pentamer.

**Fig. 3.** Sequence of an reverse transcription-polymerase chain reaction-generated COMP cDNA fragment from control ligament cells (left lanes) and patient R91-213A chondrocytes (right lanes). An arrowhead indicates the mutant nucleotide. Both alleles are present in similar levels in the cells.
Under these conditions too, strong intracellular staining was detected inside patient chondrocytes, but not controls, indicating that the RER inclusions contain COMP (Fig. 5). A similar result was obtained with cartilage from two other PSACH patients (R68-29 and R92-58) (not shown). Immunostaining with antibodies against other extracellular matrix proteins showed strong intracellular staining of patient cartilage with anti-type IX collagen antibodies, but anti-aggrecan or anti-type II collagen antibodies did not stain above control levels (Fig. 5).

DISCUSSION

COMP is a pentameric glycoprotein of the cartilage extracellular matrix responsible, when abnormal, for two autosomal dominant human skeletal dysplasias, PSACH and MED. COMP expression is limited to few, poorly accessible, tissues. To get insight in the function of COMP and to understand the cellular pathology of PSACH and MED, we have established an in vitro cell culture model to study the metabolism of the normal and mutant proteins.

Pentameric COMP Is Secreted by Control Cultured Cells—Chondrocytes isolated from adult or fetal cartilage, grown in monolayer, synthesized and secreted pentameric COMP. Since chondrocytes are known to redifferentiate quickly when grown in monolayer, results obtained in such cell cultures need to be cautiously interpreted. We therefore examined two other cell types, tendon and ligament cells, which are fibroblast-type cells. Both cell types expressed COMP, confirming, in humans, tendon as a source of this protein (19, 31). Expression of COMP by ligament cells may be relevant to the joint laxity observed in PSACH patients. Synthesis of COMP was dramatically increased by treatment of the cells with a growth factor known to affect bone and cartilage growth, TGF-β1 (28).

In all three cell types, the pentameric form of COMP was detected in the supernatant as well as inside the cells. The much greater affinity of the available antibody for the pentamer than for the monomer doesn’t allow us to rule out the presence of low levels of free monomer. However, the intracellular presence of pentamer suggests that pentamerization of COMP is an intracellular process. This result is similar to what has been observed for another protein of the thrombospondin family, thrombospondin-1, which co-translationally assembles into trimers within the lumen of the RER (32). In patients with PSACH and MED, all COMP mutations are found in the calmodulin-like or the C-terminal domain. One can therefore predict that, if patients express abnormal monomers, these may be incorporated into pentamers before the mutant region is translated and before the molecule could be detected as abnormal by the cell. This further implies that a secretion defect in patients would operate at the level of the pentamer.

COMP Is Secreted by Patient Cells—We subsequently tested patient cells of similar tissue origin with the goal of studying how the mutations affect the metabolism and function of COMP. Since MED and PSACH are inherited in an autosomal dominant mode, the pathologies could be caused by either haploinsufficiency or a dominant negative effect. Because both the normal and mutant alleles were expressed at similar levels in the patient cell cultures, we expect that the mutant RNA is stably expressed and likely to be translated. This suggests that the cells are not functionally haploinsufficient for COMP and that the mutations are pathologic, at least in part, through a dominant negative effect, due to incorporation of one or more abnormal monomers into COMP pentamers. In addition, we observed no dramatic quantitative secretion defect of COMP in cultured cells from patients with COMP mutations. This suggests that the cells in culture are able to secrete abnormal pentamers, supporting the hypothesis that COMP mutations in PSACH and MED exert their phenotypic effect through a dominant negative mechanism. If this also proves true in vivo, it implies that abnormal COMP is present in the matrix, but is not able to perform its normal function.

The biochemical data supporting a dominant negative model are also compatible with the molecular genetic data. A priori, haploinsufficiency is an unlikely mechanism, since it would be difficult to understand how different point mutations in the COMP gene could cause two distinct phenotypes (9). Also, truly genetically haploinsufficient patients have never been described. In a panel of PSACH patients in which all mutations have been identified, none had either nonsense mutations predicted to lead to a truncated protein or genomic deletion of COMP.4 It is entirely plausible that the different mutations could affect different

4 D. H. Cohn, M. D. Briggs, and L. M. King, manuscript in preparation.
aspects of the function or structure of COMP, for instance altering COMP-matrix interactions or COMP-chondrocyte binding, and produce different phenotypic consequences.

**Cultured Cells Mimic the Cellular Pathology of Cartilage in Patients**—We observed that cultured patient cells show RER inclusions, similar to those observed *in vivo*, after several

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**FIG. 5. Immunohistochemistry on cartilage.** Immunohistochemical staining of cartilage from a control fetus (left panels: A, C, E, and G) and from PSACH patient R94-291A (right panels: B, D, F, and H). Staining with polyclonal anti-COMP antibodies at a 1/1000 dilution (A, B), with anti-type IX collagen antibodies (C, D), with anti-aggrecan antibodies (E, F), and with anti-type II collagen antibodies (G, H). Magnification is the same in all pictures (× 200).
weeks in culture. These data suggest that, in addition to the dominant negative effects of COMP mutations, there is also impaired secretion of the abnormal pentamers and therefore a reduction of the amount of COMP in the extracellular matrix. Staining of the retained material with antibodies against COMP and type IX collagen has been demonstrated in one recently reported PSACH patient (23) and in three PSACH patients with COMP mutations described here. Thus intracellular retention of both COMP and type IX collagen is a general characteristic of PSACH, and reduced amounts of these two proteins in the matrix may contribute to the disease. Absence of inclusion staining with anti-aggrecan and anti-type II procollagen antibodies in our patients suggests that retention is specific and that COMP retention does not lead to a general deficit in protein secretion by chondrocytes.

Although our data, both in vitro and in vivo, identify RER inclusions in chondrocytes, there also appears to be some anti-COMP antibody staining of the cartilage matrix of patients. This suggests that the abnormal COMP allele contributes to the matrix in vivo. While this finding may be in contrast to data suggesting absence of COMP in the cartilage matrix of one PSACH patient (23), differences in antibodies and techniques may provide an explanation for the discrepancy. Another interesting finding of the paper by Maddox et al. (23) is the fact that, in contrast to chondrocytes, patient tendon cells seem to be able to secrete COMP into the extracellular matrix. It will be critical, when more tendon tissue from patients becomes available, to determine by electron microscopy whether tendon shows RER inclusions similar to those in cartilage.

In conclusion, our current model for the pathology of the COMP disorders is the combined effects of abnormal COMP in the matrix and poor secretion of abnormal pentamers by chondrocytes and other cell types that express COMP. At least in vitro, and possibly in vivo, inclusions in the RER appear to develop slowly over time. We hypothesize that retention of structurally abnormal COMP leads to secondary retention of other matrix proteins, including type IX collagen, perhaps because they normally interact with COMP. This possibility was also suggested by the observation that MED can also result from mutations in COL9A2 (11, 33). Determining the relative importance of the quantitative and qualitative defects in COMP biosynthesis on disease pathology will require a better understanding of the role of COMP in the matrix and how its specific functions are disrupted by mutations. The availability of cultured cells as an in vitro model can now be used to explore the various functions of COMP, including calcium binding, interaction with chondrocytes, and binding to other extracellular matrix components, as well as the effect of mutations on these properties.

Acknowledgments—We thank Drs. D. Heinegård and R. Poole for providing valuable antibodies; Dr. D. Krakow (Los Angeles, CA) for obtaining the tissues used to derive cell lines from fetal controls; Dr. W. G. Cole (Toronto) for alerting us to the observation by A. Recklies and M. Priore for their help at the International Skeletal Dysplasia Registry.

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