Disruption of Extracellular Matrix Structure May Cause Pseudoachondroplasia Phenotypes in the Absence of Impaired Cartilage Oligomeric Matrix Protein Secretion*\[S\]

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Pseudoachondroplasia and multiple epiphyseal dysplasia are two dominantly inherited chondrodysplasias associated with mutations in cartilage oligomeric matrix protein (COMP). The rarely available patient biopsies show lamellar inclusions in the endoplasmic reticulum. We studied the pathogenesis of these chondrodysplasias by expressing several disease-causing COMP mutations in bovine primary chondrocytes and found that COMP-associated chondrodysplasias are not exclusively storage diseases. Although COMP carrying the mutations D469\(\Delta\) and D475N was retained within the endoplasmic reticulum, secretion of COMP H587R was only slightly retarded. All pseudoachondroplasia mutations impair cellular viability and cause a disruption of the extracellular matrix formed in alginate culture irrespective of the degree of cellular retention. The mutation D361Y associated with the clinically milder disease multiple epiphyseal dysplasia gave mild retention and limited matrix alterations, but the transfected cells showed normal viability. The effect of mutated COMP on matrix formation and cell-matrix interaction may be a major element in the pathogenesis of COMP-associated chondrodysplasias.

Pseudoachondroplasia (PSACH)\[3\] and multiple epiphyseal dysplasia (MED) are two autosomal dominantly inherited forms of chondrodysplasia characterized by severe to moderate disproportionate dwarfism and pronounced joint laxity. In these patients premature osteoarthritis frequently requires early joint replacement (1). Mutations in cartilage oligomeric matrix protein (COMP) are linked to PSACH and to some forms of MED (2, 3). Mutations in the genes coding for the \(\alpha_1\), \(\alpha_2\), and \(\alpha_3\) chains of collagen IX or for matrilin-3 have also been associated with the clinically milder disease, MED (4–8). Electron microscopy of patient biopsies showed that mutated COMP is retained in typical granular or lamellar inclusions in the endoplasmic reticulum (ER) of chondrocytes (9–12). The accumulation of COMP leads to a coretention of other matrix components like collagen IX and matrilin-3 (11, 13, 14). However, the trafficking and secretion of collagen II are not affected (13, 14).

Previous studies have suggested that PSACH and MED are storage diseases of the ER (11–20). It has been proposed that the intracellular accumulation of mutated protein induces an unfolded protein response that in turn leads to apoptosis (17, 19). The reduced cellular viability could explain the growth failure of affected patients (21). Additionally, a dominant interference of mutated proteins with matrix assembly (18) and collagen fibrillogenesis (22) might explain the early joint failure and ligament laxity.

In vitro studies using recombinantly expressed COMP have demonstrated that the most common mutation in patients, D469\(\Delta\), affects calcium binding (23–25). The reduced binding capacity interferes with calcium-dependent folding and as a consequence affects the interaction of COMP with collagens (25–27).

We have previously established a cell culture model of PSACH using the COMP mutation D469\(\Delta\) (14). In the present study, we used this experimental approach to examine the role of a set of different COMP mutations in the pathogenesis of PSACH and MED to determine if the mechanism of action was identical or differed.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—All COMP constructs were expressed in a cassette consisting of the cytomegalovirus (CMV) immediate early promoter controlling the expression of a fusion protein of the BM-40 signal peptide, an N-terminal His c-myc factor X tag (28) and full-length rat COMP (see Fig. 1) (25). The pRC/CMV vector (Invitrogen) was used for plasmid-based experiments. The mutations D361Y, D469\(\Delta\), and H587R were introduced by site-directed mutagenesis as previously described (25, 29). The D475N mutation was introduced by...
PCR amplification of the type 3 domain using primer 4 (5-gccgcgcgggccgccgagc-cgccgag-cgtg-3) with primer 475m (5-ctgtcaggta-cgccgag-cgtg-3) and primer M2 (5-ccacagc-3). A silent mutation created an additional KpnI site used to identify mutant clones. The resulting cDNA was cloned into the pCEP-Pu vector (30) carrying the wild-type COMP cDNA as previously described (25, 29). Constructs were then transferred into the pRC/CMV plasmid using NheI/NotI restriction sites. The identity of all constructs was confirmed by sequencing.

Adenoviral Constructs—The AdEasy-vector system (Qbiogene) was used to generate adenoviral constructs (31). The His-c-myc-tagged COMP constructs were cloned from the pCEP-Pu plasmids generated here or in previous studies (25, 29) into the pShuttleCMV transfer vector using KpnI and XhoI or Nhel and NotI sites. This vector was linearized with Pmel, gel-purified, and extracted with phenol/chloroform. The vector pAdEasy-1 was purified by cesium chloride gradient centrifugation and cotransformed with the linearized pShuttleCMV into electrocompetent B1583 bacterial cells (Qbiogene), where homologous recombination between both plasmids took place. Resulting clones were screened for recombination by BamHI digestion. The DNA of positive clones was amplified in ultrade- X10-Gold bacterial cells (Stratagene, La Jolla, CA). Control digests were performed with BamHI, HindIII, XhoI, NotI, Nhel, Pael, KpnI, SnaI, and AclI to ensure the presence of the insert and the stability of the recombinant DNA. Recombinants were digested with Pael, purified by phenol/chloroform, and transfected into QBI-293 cells using FuGene6 (Roche Applied Science, 5 µg of DNA with 15 µl of FuGENE for 1.5×10⁶ cells). The adenoviruses were serially amplified until 10¹² viral particles were obtained. These were purified by cesium chloride gradient centrifugation, and CsCl was removed by dialysis before storage of the adenovirus at -80 °C. Viral infectious titers were determined using plaque assay and tissue culture infectious dose 50 (viral dilution, at which 50% of wells contain growing virus) methods in triplicate experiments. The resulting titer was verified using a Southern hybridization technique (32) and ranged from 2×10⁸ to 4×10¹⁰ infectious particles/ml with good concordance of the Southern hybridization technique with the tissue culture infectious dose 50. Titers obtained with plaque assays were 10- to 100-fold lower.

Culture of Primary Bovine Chondrocytes—Primary bovine articular chondrocytes were isolated by enzymatic digestion of cartilage from shoulder joints of 18- to 24-month-old animals as described earlier (33). Chondrocytes were cultured in monolayer and transfected 5 days after isolation. Cellular viability and COMP secretion was monitored 3–6 days after transfection. For long term experiments, cells were transfected with 20 m.o.i. using 8×10⁶ chondrocytes in 500 µl of serum-free medium, directly after isolation if not otherwise indicated. The suspension was incubated for 1 h. Cells were then pelleted and resuspended in 1.2% alginate for gel culture (14, 33).

Transfection and Transduction of Chondrocytes with Wild-type and Mutated COMP Constructs—Plasmids encoding for wild-type and mutated COMP constructs were transfeceted into primary chondrocytes with FuGene6 using previously established conditions (34). Adenoviral infection was performed with 20 m.o.i. using 8×10⁹ chondrocytes in 500 µl of serum-free medium, directly after isolation if not otherwise indicated. The suspension was incubated for 1 h. Cells were then pelleted and resuspended in 1.2% alginate for gel culture (14, 33).

XTT Assay—The cellular viability was determined using XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[phenylamino]carbonyl]-2H-tetrazolium hydroxide) in a microculture assay. XTT is metabolized by living cells to a formazan product, which is soluble in water and detectable by photometry. To obtain a more efficient reduction of XTT by the cells, phenazine methosulfate (Sigma) was added to the assay as described earlier (35). Cells were cultured in 6-well plates, and the XTT assay was performed 6 days after transfection. After 4 h of incubation, the optical density was measured at 450 nm.

SDS-PAGE and Immunoblotting—SDS-PAGE was performed as described by Laemmli (36). Secretion of recombinant proteins was analyzed by SDS-PAGE of cell culture supernatants and cell extracts, obtained by sonication, followed by immunoblotting. Samples were concentrated by ethanol precipitation. For immunoblots, proteins were transferred to nitrocellulose and incubated with a mouse antibody directed against the c-myc epitope (9E10, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in Tris-buffered saline containing 5% low fat milk powder. Bound antibodies were detected by luminescence using horseradish peroxidase-conjugated rabbit antimouse IgG (DAKO), 1.25 mm 3-aminophthalhydrazide, 225 mm p-coumaric acid, and 0.01% H₂O₂.

Metabolic Labeling of COMP—The kinetics of COMP secretion was assessed in pulse-chase experiments. Cells in monolayer were starved for 2 h in cysteine- and methionine-free medium, pulsed for 1 h with medium containing 50 µCi/ml of a [³⁵S]methionine/cysteine labeling mix (PerkinElmer Life Sciences), and chased in normal medium. After harvest of culture supernatants, monolayer cells were lysed in 1% Nonidet P-40, 50 mM Tris, pH 7.4 (lysis buffer). Alginic beads were analyzed after 4 weeks of culture. Beads were starved over night in cysteine- and methionine-free medium, pulsed for 4 h with medium containing 50 µCi/ml of a [³⁵S]methionine/cysteine labeling mix (PerkinElmer Life Sciences), and chased in normal medium for 72 h. Supernatants were harvested, and the beads were dissolved in 55 mm sodium citrate and 150 mm sodium chloride as described previously (33). The citrate-resistant fraction was treated with lysis buffer. Supernatants, citrate-soluble fractions, and the lysed citrate-resistant fraction were immunoprecipitated as described below.

Immunoprecipitation of COMP—A mouse monoclonal antibody directed against the c-myc epitope (9E10, Santa Cruz Biotechnology) was coupled to protein A-agarose for 4 h at room temperature. Unspecific binding was blocked with 10% bovine serum albumin, and samples were precleared with protein A-agarose. The antibody-protein A beads were added, and the mixtures were incubated overnight at 4 °C. After precipitation, the beads were washed three times with 1% Triton X-100 in
After fixation with 2% paraformaldehyde and permeabilization using 0.2% Triton X-100 in phosphate-buffered saline, the cultures were treated with 2% normal goat serum in phosphate-buffered saline as blocking reagent. The cells were incubated with primary antibodies for 1 h, followed by incubation with a secondary antibody for 1 h. Primary antibodies directed against protein disulfide isomerase (Biomol), a marker for the ER, and 58K (Sigma), a marker for the Golgi apparatus, were used together with a polyclonal rabbit antibody directed against the c-myc epitope (Santa Cruz Biotechnology), visualizing transgenic COMP, followed by secondary Cy3- and Alexa 488-conjugated antibodies against mouse or rabbit IgG (Molecular Probes). The slides were finally mounted in DAKO fluorescent mounting medium and evaluated with a confocal laser scanning microscope (Leica TCS SP) using krypton and argon lasers. Alginate beads were embedded in paraffin, processed, and stained as described previously (33), using the monoclonal mouse antibody 9E10 against the c-myc epitope.

**Analysis of Collagen Synthesis**—Alginate cultures were analyzed 4 weeks after transduction with 20 m.o.i. of virus. On day −7, fresh complete medium containing 5 μCi/ml of [14C]proline was added. On days −5 and −3, the supernatant was harvested and replaced by fresh medium of identical composition. On day 0, the harvested supernatants were combined, and the alginate beads were dissolved as described above. Equal proportions of the supernatant and the citrate-soluble matrix were digested with a secondary antibody for 1 h. Primary antibodies directed against protein disulfide isomerase (Biomol), a marker for the ER, and 58K (Sigma), a marker for the Golgi apparatus, were used together with a polyclonal rabbit antibody directed against the c-myc epitope (Santa Cruz Biotechnology), visualizing transgenic COMP, followed by secondary Cy3- and Alexa 488-conjugated antibodies against mouse or rabbit IgG (Molecular Probes). The slides were finally mounted in DAKO fluorescent mounting medium and evaluated with a confocal laser scanning microscope (Leica TCS SP) using krypton and argon lasers. Alginate beads were embedded in paraffin, processed, and stained as described previously (33), using the monoclonal mouse antibody 9E10 against the c-myc epitope.

**Immunogold Labeling**—Alginate beads were incubated overnight in 2% glutaraldehyde, 0.5% paraformaldehyde, 3 mM calcium chloride, 0.1 M cacodylate, pH 7.4. The beads were dehydrated in a graded ethanol series and finally embedded in LR gold (Fluka). The polymerization took place at −25 °C for 24 h under UV light. For immunoreaction, ultrathin sections were incubated with a gold-labeled c-myc antibody for 1 h at room temperature. Contrasting and viewing was as described above.

**Cell Attachment Assay**—Primary bovine chondrocytes were isolated as described above, and COMP variants were expressed and purified as described earlier (25, 29). 96-well plates were coated overnight at 4 °C with purified proteins. Unspecific binding sites were blocked with 1% bovine serum albumin. 5 × 10^4 chondrocytes were incubated for 20 h at 37 °C in substrate-coated wells as described earlier (37). Non-attached cells were removed by careful washing with phosphate-buffered saline. Attached cells were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet for 30 min. The excess dye was washed off and after addition of 0.2% Triton X-100, the plate was measured in an enzyme-linked immunosorbent assay reader at 562 nm.

**RESULTS**

COMP carrying mutations associated with PSACH or MED was expressed in primary bovine chondrocytes after plasmid-based and adenoviral gene transfer. All constructs encoded an N-terminal His c-Myc tag to allow the discrimination between transgenic and endogenous COMP (Fig. 1). The mutation D469A was chosen because it is the most frequent PSACH-associated COMP mutation (3, 38) localized in the thrombospondin type 3 domain. Previous studies have characterized the consequences of this mutation in vitro (13, 14, 23–27). The mutation D361Y was for comparison, because it is also an aspartate exchange located in the type 3 domain and is associated with the milder disease form MED (39). H587R is particularly interesting, being one of the very few PSACH-associated COMP mutations within the putative collagen binding site in the C-terminal domain (27, 40). To complement the analysis of PSACH-associated COMP mutations, D475N, another mutation in the type 3 domain that is not associated with an amino acid deletion (40, 41), was cloned using the approach described previously (25, 29).
Immunoblotting of cell culture supernatants and lysates of plasmid-transfected cells (Fig. 2A) revealed that for two of three PSACH-associated COMP mutations (D469Δ and D475N) most COMP was detected within the cell lysates. The third PSACH-associated mutation, H587R, the MED-associated mutation D361Y, and the transgenic wild-type protein were mainly found in the cell culture supernatant, suggesting that the secretion of D469Δ and D475N, but not of D361Y and H587R, is markedly impaired. In each case, the proteins migrated as pentamers in SDS-PAGE. Similar results were obtained when cells were adenovirally transduced and the protein secretion was analyzed after 16 h by a pulse-chase experiment (Fig. 2B). Although the absolute amount of a certain COMP variant recovered could vary between experiments, reflecting slight differences in gene transfer efficiency, all experiments showed more pronounced retention of D469Δ and D475N than of the other mutants. These results were supported by quantitative pulse-chase experiments (Fig. 2C). The sum of the radioactivity in supernatant and cell lysate at a given time point was set as 100%, and the proportion of intracellular COMP was blotted against the time (Fig. 2D). This revealed that secretion of the MED-associated mutated protein D361Y and of the PSACH-associated mutation H587R was only moderately delayed as compared with transgenic wild-type protein; the retention observed after 24-h chase was not as pronounced as for mutations D469Δ and D475N.

Immunofluorescence microscopy of both plasmid-transfected and adenovirally infected cells showed that most of the COMP D469Δ and D475N proteins colocalized with protein disulfide isomerase, a marker for the ER (Fig. 3 and supplemental Fig. S1). In contrast, transgenic wild-type COMP and the mutant H587R were mainly found in the Golgi compartment, visualized using an antibody directed against the 58K protein. The mutant protein D361Y showed an intermediate behavior with some costaining with protein disulfide isomerase but also a pronounced signal in the Golgi (Fig. 3).

Expression of all PSACH-associated COMP mutations, i.e. D469Δ, D475N, and H587R, led to a decrease in cellular viability, irrespective of the degree of intracellular retention, as assessed by a tetrazolium salt assay after plasmid-based gene transfer. The transgenic expression of wild-type COMP or of the MED-associated mutation D361Y did not decrease viability (Fig. 4). Similar effects on cell viability were observed after adenoviral infection (results not shown).

To assess the extracellular matrix formed, chondrocytes were cultured in alginate beads for 4 weeks after adenoviral gene transfer. Immunohistochemistry revealed a strong transgenic COMP signal in the pericellular matrix of cultures transduced with wild-type COMP (Fig. 5). Transgenic COMP could be detected in almost all cell clusters as long as 4 weeks after gene transfer, indicating a highly efficient infection and long-term expression of COMP. All cell clusters displayed a strong pericellular COMP signal. Many cells expressing the two retained PSACH-associated mutated COMP proteins D469Δ and D475N stained in a pattern suggestive of intracellular retention, whereas most cells expressing wild-type COMP and
the mutation H587R displayed a predominantly extracellular staining (Fig. 5).

We then performed experiments to more closely characterize the matrix formed by transduced chondrocytes after prolonged culture in alginate. Metabolic labeling of collagens with \[^{14}\text{C} \] proline showed no difference between cultures in the amount and distribution of cross-linked collagens or of the pepsin-digested α1 chain of collagen II. Even after 4 weeks of culture, no collagen I was observed (results not shown). A quantitative analysis of the distribution of radioactive collagen II between the culture supernatant, the citrate soluble alginate matrix, and the citrate-resistant matrix pellet did not reveal any striking differences in the distribution of collagen II between chondrocytes expressing different forms of COMP constructs (Fig. 6A). We also determined the relative distribution of \[^{35}\text{S} \] methionine/cysteine-labeled COMP in the three matrix fractions at steady state. In untransduced control cultures and in cultures expressing exogenous wild-type COMP, the lysed citrate-resistant pellet corresponding to the pericellular matrix, and the intracellular compartment contained equivalent amounts of COMP compared with the citrate-soluble matrix (Fig. 6B). The mutated D361Y and H587R proteins were distributed between compartments in a manner similar to wild-type COMP. In contrast, proportionally more COMP was detected in the citrate-resistant pellet compared with the citrate-soluble matrix in cultures expressing the PSACH-associated mutated COMP proteins D475N and, to a lesser degree, D469Δ.

Electron microscopy showed a severely altered extracellular matrix in all cultures expressing PSACH-associated COMP mutations irrespective of their secretion characteristics. The matrix of untransduced cultures and such expressing wild-type COMP contained abundant collagen fibrils with some electron-
Pathogenesis of PSACH Is Heterogenous

FIGURE 5. Immunohistochemical staining of chondrocytes cultured in alginate beads for 4 weeks after adenoviral transduction with 20 m.o.i. Cultures were stained with an antibody directed against c-Myc. A c-Myc signal is detected in the pericellular matrix around almost all chondrocytes (arrows). Cultures expressing COMP carrying mutations that cause ER retention also show staining suggestive of intracellular retention (arrowheads). Cell borders are outlined with a white line. wt, transgenic wild-type COMP; ΔD, D469Δ; DN, D475N; and HR, H587R. The bar represents 10 μm.

dense spots corresponding to proteoglycans associated with the fibrils (Fig. 7). The matrix formed after expression of the MED-associated COMP mutant D361Y was moderately altered with slightly disturbed collagen fibrils. Occasionally, diffuse electron-dense aggregates were present. The matrix formed by cultures expressing the PSACH-associated COMP mutants D469Δ, D475N, and H587R was characterized by abundant diffuse electron-dense aggregates and a disruption of collagen fibril formation compared with untransduced control cultures or wild-type cultures (Fig. 7). Immungold labeling with the antibody against c-Myc confirmed that the electron-dense aggregates contain mutated COMP (Fig. 7) as previously shown for mutated D469Δ (16). We did not observe any large intracellular inclusions in the ER of several samples analyzed (not shown).

H587R COMP did not induce severe trafficking defects but had a pronounced effect on cellular viability. Therefore, we asked whether the secretion and deposition of mutated COMP in the disrupted matrix might signal back to the cells. To address this question, we coated recombinantly expressed protein onto microwell plates and performed cell attachment assays with freshly isolated primary chondrocytes. Chondrocytes showed a protein dose-dependent attachment to both fibronectin and wild-type COMP or mutated COMP (Fig. 8A). Light microscopy revealed that cell spreading took place in fibronectin-coated wells and is initiated in wells coated with wild-type COMP after 20 h. Coating with H587R and particularly D469Δ COMP reduced cell attachment significantly as compared with wild-type COMP (Fig. 8B).

D469Δ causes less disruption of the extracellular matrix. Retention of mutated COMP and the formation of COMP-containing lamellar inclusions of the ER are considered the main pathogenic features of pseudoachondroplasia (9–13, 15–19). Although we observed the expected delayed secretion of the mutations D475N and D469Δ in the present study we did not find the granular inclusions of the ER, previously described in this model (14). The lack of inclusions can be explained by the lower infectious titer used (20 instead of 100 m.o.i.), the lower viral dose giving less synthesis of transgenic COMP. The fact that the disruption of the collagen network is apparent in the absence of accumulation of COMP in the ER suggests that the deleterious effect of mutated COMP on extracellular matrix assembly has been underestimated, although it has been observed in rat chondrosarcoma cells and chondrocyte cultures from patients expressing the D469Δ mutation (13, 18). The mutation H587R demonstrates that in certain cases this pathogenic pathway might even be more important than the ER retention. However, in case of a matrilin-3 mutation that leads to intracellular protein accumulation within the ER, the ultrastructure of the matrix was not affected (43). Studies of cell attachment to wild-type and mutant forms of COMP (Fig. 8) indicated another mechanism by which COMP mutations may influence chondrocyte viability and function. Both the well secreted mutant H587R and the mutant D469Δ, which is strongly retained in chondrocytes, supported attachment less efficiently than the wild-type protein. Thus we have evidence that patient-derived mutations, causing moderate alterations in protein

DISCUSSION

This study shows that the pathogenic pathways of COMP-associated chondrodysplasias are more heterogeneous than previously appreciated (11, 12, 14, 42). Of three COMP mutations associated with the severe phenotype of PSACH, two (D469Δ, D475N) cause severe protein retention within the ER of articular chondrocytes, whereas trafficking and secretion of one (H587R) is less affected. Although both the D361Y and H587R mutant proteins displayed a slightly delayed secretion in pulse-chase experiments, long term cultures in alginate demonstrate that this does not lead to a pronounced accumulation of the proteins in the ER. Interestingly, all three PSACH-associated mutations cause a decrease in cellular viability and lead to a similar disruption of the extracellular matrix formed in three-dimensional culture. The MED-associated COMP mutation D361Y, which is retained to a lesser degree than D475N and D469Δ, causes less disruption of the extracellular matrix.

Retention of mutated COMP and the formation of COMP-containing lamellar inclusions of the ER are considered the main pathogenic features of pseudoachondroplasia (9–13, 15–19). Although we observed the expected delayed secretion of the mutations D475N and D469Δ in the present study we did not find the granular inclusions of the ER, previously described in this model (14). The lack of inclusions can be explained by the lower infectious titer used (20 instead of 100 m.o.i.), the lower viral dose giving less synthesis of transgenic COMP. The fact that the disruption of the collagen network is apparent in the absence of accumulation of COMP in the ER suggests that the deleterious effect of mutated COMP on extracellular matrix assembly has been underestimated, although it has been observed in rat chondrosarcoma cells and chondrocyte cultures from patients expressing the D469Δ mutation (13, 18). The mutation H587R demonstrates that in certain cases this pathogenic pathway might even be more important than the ER retention. However, in case of a matrilin-3 mutation that leads to intracellular protein accumulation within the ER, the ultrastructure of the matrix was not affected (43). Studies of cell attachment to wild-type and mutant forms of COMP (Fig. 8) indicated another mechanism by which COMP mutations may influence chondrocyte viability and function. Both the well secreted mutant H587R and the mutant D469Δ, which is strongly retained in chondrocytes, supported attachment less efficiently than the wild-type protein. Thus we have evidence that patient-derived mutations, causing moderate alterations in protein
structure, can extracellularly affect both matrix assembly and cell-matrix interactions. However, we do not yet have formal proof that these changes are the mechanisms causing patient symptoms. Such proof may come from future in vivo studies in genetically modified animals.

The mutations D361Y, D469/H9004, and D475N used in our study are localized within the thrombospondin type 3 domain and alter calcium binding and protein structure (23–26, 44, 45). The mutation H587R is found in the C-terminal globular domain within a putative collagen binding site (27, 29). In vitro, this mutation did not cause significant alterations of COMP structure and collagen binding (29). The most prevalent mutations occur in the thrombospondin type 3 domain (26, 40, 45) and most clinical studies probably describe the intracellular phenotype caused by mutations in this domain. Although a sporadic-case patient carrying the H587R mutation suffered from typical

**FIGURE 7.** Electron microscopy of the matrix formed by chondrocytes in alginate cultures 4 weeks after adenoviral gene transfer with 20 m.o.i. Left panels: untransduced cultures and cultures expressing transgenic wild-type COMP; DY, D361Y; ΔD, D469Δ; DN, D475N; and HR, H587R. The bars correspond to 200 nm.
Pathogenesis of PSACH Is Heterogenous

PSACH (40), cartilage biopsies were not studied. We lack a histological description of the patient cellular phenotype caused by this or other mutations in the C-terminal domain.

Our experimental approach allows a direct comparison of pathogenic mechanisms of different matrix protein mutations, irrespective of their clinical frequency, the heterogeneity of clinical presentation, and sampling of human material. A potential drawback of our model system is that it employs gene transfer and overexpression of a mutated protein to study within weeks a human disease that takes months or years to develop. The validity of our model is, however, supported by the observation that cultures overexpressing wild-type COMP do not show anomalies in matrix formation compared with untransduced cultures and that the expression of a COMP mutation associated with mild chondrodysplasia leads to only moderate matrix alterations and a mild cellular phenotype.

A recent report showed that the secretion rate of COMP carrying mutations in the type 3 domain in 293 EBNA cells is affected by the signal peptide (46). Targeting to the secretory pathway by the COMP signal peptide delayed the secretion of mutant and wild-type COMP when compared with the BM-40 signal peptide used in our current and previous studies. However, even with the COMP signal peptide, the D469Δ protein investigated was eventually secreted. It is possible that secretion of H587R COMP might be more delayed when targeted by a different signal peptide, but it is not likely that this delay would lead to a complete stop of secretion. In addition, we find not only a similar matrix phenotype irrespective of the degree of COMP retention but also a comparable decrease in cellular viability when expressing all three PSACH-associated mutant COMP proteins. The cellular viability is impaired even in the absence of COMP retention. This observation suggests that different pathways lead to reduced viability, one presumably depending on the unfolded protein response induced by retained proteins (14, 16, 19), another on extracellular mechanisms, possibly involving altered chondrocyte-matrix interactions, as demonstrated here by impaired cell attachment of primary chondrocytes to mutant COMP variants. It has been shown that chondrocytes bind to COMP through receptors of the integrin family (47) and that integrin-mediated interactions with collagen II are important for chondrocyte survival (48–50). It is conceivable that either an altered binding of COMP to integrin receptors or the COMP-mediated disruption of the extracellular matrix may induce apoptosis.

In summary, we present evidence that COMP retention is not always the cause of the PSACH phenotype. Irrespective of their rate of secretion, all PSACH-associated mutant COMP proteins investigated impair cellular viability and disrupt the extracellular matrix, thus presumably causing the growth failure and early joint destruction seen in affected patients.

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FIGURE 8. Attachment of primary chondrocytes to fibronectin and purified COMP variants. A, chondrocytes were allowed to attach to wells coated with the indicated concentrations of either fibronectin (closed circles), wild-type (closed rhombus), D469Δ (open circles), and H587R COMP (open squares). Nonspecific cell adhesion was determined by plating cells onto wells coated with bovine serum albumin, and this value was subtracted from the experimental data. B, morphology of crystal violet-stained chondrocytes attached to 10 μg/ml protein was evaluated after 20 h by light microscopy.
