Correction of Abnormal Matrix Formed by cmd/cmd Chondrocytes in Culture by Exogenously Added Cartilage Proteoglycan

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Abstract. The cartilage matrix deficiency (cmd/cmd) mouse fails to synthesize the core protein of cartilage-characteristic proteoglycan (cartilage PG). Chondrocytes from the cmd/cmd cartilage cultured in vitro produced nodules with greatly reduced extracellular matrix. Immunofluorescence staining revealed that the nodules of mutant cells differed from the normal in lacking cartilage PG and in uneven and reduced deposition of type II collagen. Exogenously added cartilage PG prepared from either normal mouse cartilage or Swarm rat chondrosarcoma to the culture medium was incorporated exclusively into the extracellular matrices of the nodules, with a concurrent correction of the abnormal distribution pattern of type II collagen. The incorporation of cartilage PG into the matrix was disturbed by hyaluronic acid or decasaccharide derived therefrom, suggesting that the incorporation process involves the interaction of added proteoglycan with hyaluronic acid. Both the hyaluronic acid–binding region and the protein-enriched core molecule prepared from rat chondrosarcoma cartilage PG could also be incorporated but, unlike the intact cartilage PG, they were distributed equally in the surrounding zones where fibroblast-like cells predominate. The results indicate that the intact form of cartilage PG is required for specific incorporation into the chondrocyte nodules, and further suggest that cartilage PG plays a regulatory role in the assembly of the matrix macromolecules.

THE extracellular matrix of cartilage is composed primarily of cartilage-characteristic proteoglycan (cartilage PG)[1] and type II collagen. Cartilage PG is present in the matrix as high molecular weight aggregates in which proteoglycan monomers bind specifically to hyaluronic acid at regular intervals through stabilization by link protein (8). Type II collagen molecules also are assembled to form fibrils with characteristic size and morphology (for review, see reference 33).

Cartilage matrix deficiency (cmd/cmd) is an autosomal recessive lethal mutation in mice resulting in a syndrome including disproportionate dwarfism, short snout, and cleft palate (26). These abnormalities have been shown to result from failure in the synthesis of cartilage PG core protein (15). Other matrix components, such as type II collagen, small proteoglycans, hyaluronic acid, and link protein are synthesized nearly at normal rates (3, 15, 18). Therefore, the cell culture of cmd/cmd chondrocytes provides a useful system in which the function in matrix assembly of cartilage PG may be studied.

We show here that cmd/cmd chondrocytes in monolayer culture exhibit an abnormal extracellular matrix and that the abnormality could be corrected by adding cartilage PG to the culture medium. Evidence is presented to show that the exogenous cartilage PG is integrated into the matrix of mutant chondrocytes through the processes in which the interactions of an intact form of cartilage PG with the other matrix molecules are involved.

Materials and Methods

Materials

Crude collagenase CLS III and fetal calf serum were purchased from Gibco, Grand Island, NY; testicular hyaluronidase (grade V) was from Sigma Chemical Co., St. Louis, MO.; Dulbecco's modified Eagle's medium and Hanks' basal salt solution were from Nissui, Tokyo, Japan; Dispase was from Godo Shusei, Tokyo, Japan; culture dishes were from Falcon Labware, Becton, Dickinson & Co., Oxnard, CA; nonfluorescence coverslips were from Matsunami Glass, Ohsaka, Japan; and [³⁵S]sulfate was from Japan Radioisotope Association, Tokyo, Japan. Rat chondrosarcoma cartilage PG, the clostripain produced hyaluronic acid–binding region, the chondroitin sulfate–rich region (4), the chondroitinase ABC–produced protein-enriched core molecule (24) derived therefrom, and cartilage PG from 12-d-old chick embryo epiphyseal cartilage (PG-H) (14) were prepared as described as previously. Fluorescein isothiocyanate–conjugated goat anti–rabbit IgG, fluo-

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1. *Abbreviations used in this paper*: cartilage PG, cartilage-characteristic proteoglycan; PG-H, cartilage PG from 12-d-old chick embryo epiphyseal cartilage.

rescein isothiocyanate–conjugated rabbit anti–guinea pig IgG, tetramethyl-rhodamine isothiocyanate–conjugated rabbit anti–guinea pig IgG, and nonimmunized goat serum were obtained from Cappel Laboratories, Cochranville, PA. Antibodies to rat chondrosarcoma cartilage PG fraction (aAl-D1) raised in a rabbit (15) were given by Dr. H.-J. Barrach (National Institute of Dental Research, National Institutes of Health, Bethesda, MD). Their specificity was established by the enzyme-linked immunosorbent assay (25) and the immunofluorescence labeling of target tissues (15, 16, 18). The antiserum had a titer of >12,000 against the injected antigen and showed little activity (titer, <50 by the enzyme-linked immunosorbent assay) against type II collagen, fibronectin, link protein, and small proteoglycans from the normal rat cartilage. The hyaluronic acid–binding region and the chondroitin sulfate–rich region prepared from cartilage PG were recognizable with the antiserum, but their antigenic activities were about one-tenth and one-fiftieth, respectively, the activity of the intact proteoglycan, as measured by the enzyme-linked immunosorbent assay. The guinea pig antibodies to rat chondrosarcoma type II collagen were a gift from Dr. J.-M. Foidart (National Institute of Dental Research, NIH). The antiserum was purified by crossimmunoadsorption and its specificity was established elsewhere (5, 16). The rabbit antibodies to rat chondrosarcoma link protein (31) and the rabbit antibodies to mouse Englebreth-Holm-Swarm tumor proteoheparan sulfate (9) were kindly given by Dr. J. H. Kimura (Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL) and Drs. J. Hassell and G. R. Martin (National Institute of Dental Research, NIH), respectively. The rabbit antibodies to PG-H were prepared as described previously (15) and their specificity was established elsewhere (28). Hyaluronic acid and its oligosaccharide derivatives were given by Mr. M. Mizutani, Seikagaku Kogyo Co., Tokyo, Japan.

Chondrocyte Culture

18- or 19-d-old embryos were obtained from proven cmd/+ × cmd/+ matings and separated into normal and cmd/cmd by their characteristic features. The epiphyseal cartilages of the embryonic knee joints were dissected free of the muscle and the ossified shaft in Hanks' basal salt solution containing 5% fetal calf serum. The cartilages were suspended in Dispase (400 U/ml) in Hanks' basal salt solution containing 5% fetal calf serum and incubated at 37°C for 30 min with gentle stirring. The perichondral layers and connective tissues surrounding the cartilages were removed by this treatment. Chondrocytes were then dissociated by incubating the cartilaginous tissues for 2 h in 0.1% (wt/vol) collagenase solution in Dulbecco's modified Eagle's medium containing 10% fetal calf serum as described by Benya et al. (1). After gentle pipetting, dissociated cells were passed through a Nitex filter (20-μm pore size) to remove aggregating cells and resuspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 U penicillin/ml, and 50 μg streptomycin/ml (medium A). About 10⁵ cells were seeded into a 35-mm Falcon tissue culture dish containing 2 ml of medium A, or on a sterilized coverslip in the same dish. Incubation was carried out at 37°C in a humidified atmosphere of 95% air/5% CO$_2$. Medium was changed every other day. Where indicated, the media were supplemented, on the second day of culture, with mouse cartilage PG (5, 10, or 50 μg/ml), mouse proteoheparan sulfate (10 μg/ml), rat chondrosarcoma cartilage PG (10 μg/ml), or chick PG-H (50 μg/ml). In experiments where inhibition of the incorporation of cartilage PG into the cmd/cmd matrix was to be examined, hyaluronic acid (50 μg/ml), oligosaccharide from hyaluronic acid (50 μg/ml), or chondroitin sulfate (50 μg/ml) was added simultaneously with the cartilage PG. When structural specificity of the proteoglycan for the incorporation ability was to be examined, the hyaluronic acid–binding region (10 μg/ml), the chondroitin sulfate–rich region (10 μg/ml), or the protein-enriched core molecule (10 μg/ml), prepared from rat chondrosarcoma cartilage PG was added to the cmd/cmd chondrocyte cultures.

Preparation of Cartilage PG

Epiphyseal cartilages were dissected from 18-d-old normal mouse embryos. The cartilages were extracted twice with 4 M guanidine-HCl in 0.2 M Tris-HCl, (pH 8.0), containing protease inhibitors (0.01 M EDTA, 0.01 M N-ethylmaleimide, 1 mM phenylmethanesulfonylfluoride, and 0.36 mM pepstatin) at 4°C for 24 h as previously described (14, 23). The extracts were centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant solution was dialyzed against 9 vol 0.2 M Tris-HCl (pH 8.0) containing the protease inhibitors. The solution was then subjected to CsCl isopycnic centrifugation under the associative condition with a starting density of 1.62 g/ml (6), in a rotor (model RP55T; Hitachi, Tokyo) at 40,000 rpm at 10°C for 50 h. The bottom third of the gradient (A1) was collected (6). The A1 fraction was subjected to CsCl isopycnic centrifugation under the dissociative condition in

4 M guanidine-HCl containing the protease inhibitors with a starting density of 1.45 g/ml as described above. The bottom third of the gradient, (A1-D1), was collected (10), dialyzed for 24 h against two 1-l changes of 0.01 M Tris-HCl (pH 7.5) and finally against distilled water for 12 h. The dialyzed solution was lyophilized, weighed, and redissolved in a small volume of the culture medium. Using the procedure of Bitter and Muir (2), the amount of proteoglycan in solution was estimated by measuring hexuronate that accounts for ~25% of the total weight of the proteoglycan (17).

Preparation of [³⁵S]Sulfate-labeled Cartilage-PG

Epiphyseal cartilages from 18-d-old normal embryos were preincubated in Dulbecco's modified Eagle's medium for 1 h. The medium was then changed to 5 ml of Ham's F12 medium containing 500 μCi of carrier-free [³⁵S]sulfate and 10% fetal calf serum. Incubation was carried out for 24 h at 37°C in a humidified atmosphere of 95% air/5% CO$_2$. Labeled cartilage PG was prepared by the same procedure as used for the preparation of unlabeled cartilage PG (see above).

Binding Experiments of Radiolabeled Cartilage PG

³⁵S-labeled cartilage PG dissolved in medium A was added on the second day of culture to the medium of either cmd/cmd or normal chondrocyte culture at the concentrations indicated in the individual experiments. Where indicated, hyaluronic acid (50 μg/ml), hyaluronic acid oligosaccharide (50 μg/ml), unlabeled rat chondrosarcoma cartilage PG (20 μg/ml), the hyaluronic acid–binding region (10 μg/ml), or the chondroitin sulfate–rich region (10 μg/ml) was added to the culture media. Incubation was carried out at 37°C for 24 h in a humidified atmosphere of 95% air/5% CO$_2$. After a 24-h incubation, the medium was collected and the cells were washed eight times with medium A, similar to the washings in the immunofluorescence staining (see below). The spent medium and the washings were combined and used as "medium fraction" for determining the amount of ³⁵S-labeled cartilage PG. The cartilage PG incorporated into the cell layer was treated with 0.2 M NaOH for 24 h at 37°C to release ³⁵S-labeled glycosaminoglycans. Radioactivity of the released glycosaminoglycans was measured and is taken to represent the quantity of ³⁵S-labeled cartilage PG in the cell layer fraction.

Labeling of Cultured Chondrocytes with [³⁵S]Sulfate for Analyses of Proteoglycan Synthesis

On the seventh day of culture, when chondrocytes yielded significant numbers of nodules, medium A containing 20 μCi of [³⁵S]sulfate/ml was added and incubation was continued for 24 h. The spent medium was collected for analysis of proteoglycans. Proteoglycans in the cell layer were extracted with 4 M guanidine-HCl containing the protease inhibitors (see above). Unlabeled rat chondrosarcoma cartilage PG (200 μg as hexuronate) was added as a carrier to each sample of the media and cell extracts, and macromolecules were recovered by precipitation with ethanol containing 1.3% (wt/vol) potassium acetate. The precipitates were dissolved in water, and the precipitation was repeated five times to ensure complete removal of radioactive low-molecular-weight compounds (13). The macromolecular fractions were dissolved in 1 ml of 4 M guanidine-HCl containing the protease inhibitors and layered on 13 ml of a linear gradient of glycerol (10–35%, wt/vol) in 4 M guanidine-HCl containing the protease inhibitors. The gradient was formed on a cushion of 0.5 ml of 50% glycerol in the same solvent (12–14). The gradient was centrifuged in a Hitachi RPS25-2 rotor at 22,500 rpm at 19°C for 28 h (12–14), and then fractionated into 0.6-ml portions. Aliquots of each fraction were assayed for radioactive proteoglycans.

Immunofluorescence Staining

Chondrocytes cultured on coverslips were rinsed with three changes of PBS (pH 7.0) and air-dried. In control experiments to see effects of air-drying fixation, samples were directly stained with antibodies without air-drying. In some experiments, the cell layers were subsequently treated with bovine testicular hyaluronidase (grade V; Sigma Chemical Co.) in PBS (0.2 mg/ml) for 15 min at 37°C and then rinsed three times. For the staining of cartilage PG or type II collagen, the samples were incubated in a humidified atmosphere for 30 min at room temperature with rabbit antibodies to cartilage PG or guinea pig antibodies to type II collagen (1:30 dilution). After rinsing with five changes of PBS, the treated samples were incubated in a humidified atmosphere with fluorescein isothiocyanate-conjugated anti–rabbit or guinea pig IgG (1:30 dilution) for another 30 min at room temperature. After washing with three changes of PBS followed by air-drying, the samples were
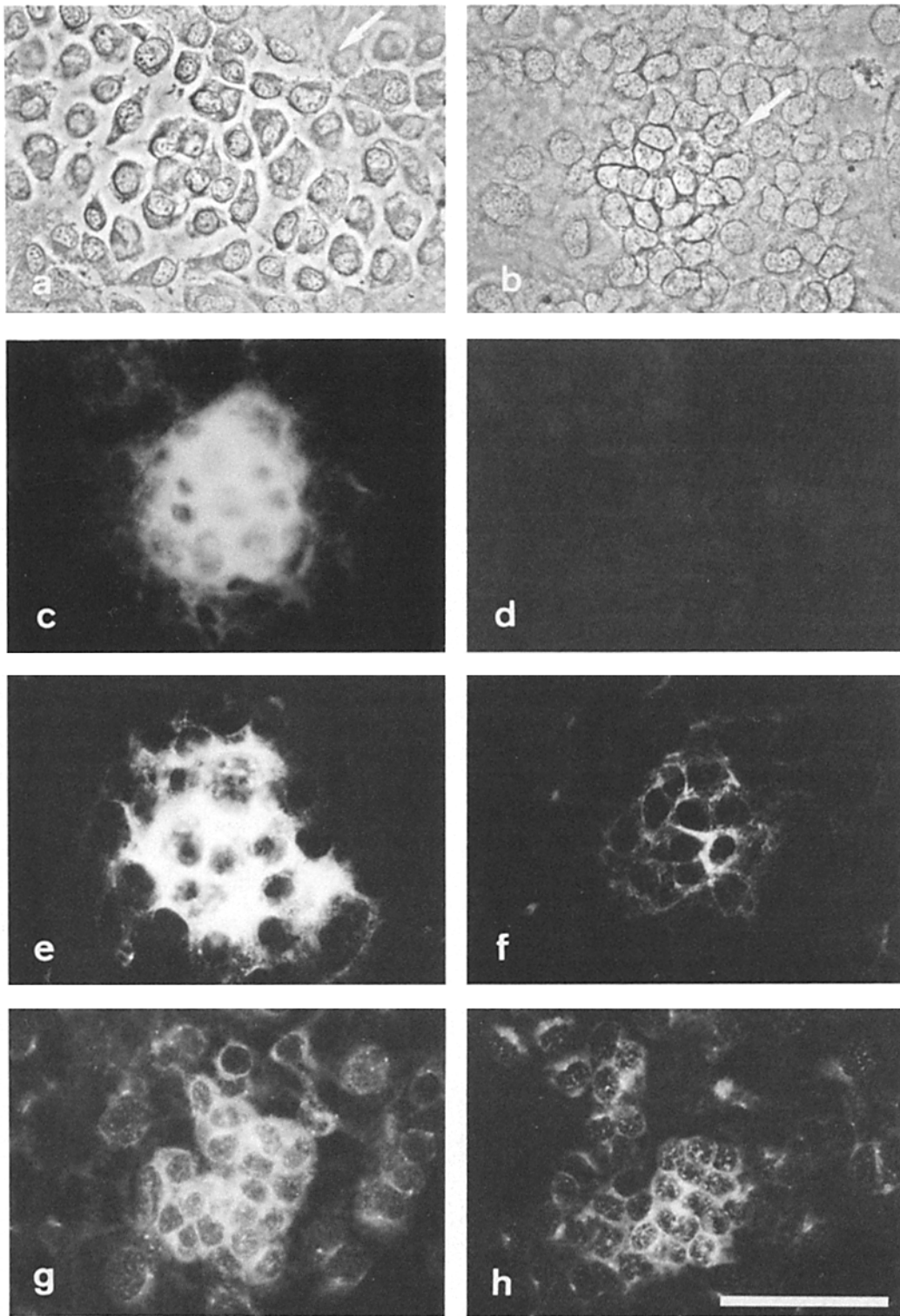
Figure 1. Morphological features and immunofluorescence staining patterns of day 7 cultures of normal chondrocytes (*left*) and mutant chondrocytes (*right*). (*a* and *b*) Phase-contrast micrographs of the cultures. The extracellular matrix is more abundant in the normal chondrocyte nodules (*arrow* in *a*) than in the mutant chondrocyte nodules (*arrow* in *b*). (*c* and *d*) Immunofluorescence staining of the cultures with antibodies to cartilage PG, (*e* and *f*) with antibodies to type II collagen, and (*g* and *h*) with antibodies to link protein. Bar, 100 µm.

mounted in buffered 90% (vol/vol) glycerol. Immunofluorescence staining with antibodies to link protein, proteoheparan sulfate, or chick PG-H were done as described above.

Double staining of cartilage PG and type II collagen was performed as follows. Samples were subjected to sequential treatments with rabbit antibodies to cartilage PG (1:30 dilution unless otherwise indicated; 30 min;

room temperature; rinsed with PBS five times), with tetramethylrhodamine isothiocyanate–conjugated goat anti-rabbit IgG (1:30 dilution; 30 min; room temperature; rinsed in PBS three times), with guinea pig antibodies to type II collagen (1:30 dilution; 30 min; room temperature; rinsed in PBS five times), and with fluorescein isothiocyanate–conjugated rabbit anti-guinea pig IgG (1:30 dilution; in the presence of nonimmunized goat serum, 1:2

dilution; 30 min; room temperature; rinsed in PBS three times). Some samples were pretreated with testicular hyaluronidase (see above) to degrade glycosaminoglycans which otherwise might mask the antigens. Fluorescence was observed under an Olympus fluorescence microscope BHK-II.

Results

Comparison of Chondrocyte Phenotypes in Culture

Application of the cell dissociation methods with trypsin resulted in a low viability of mutant chondrocytes. Using collagenase in the presence of 10% fetal calf serum for the dissociation of chondrocytes (1) greatly improved the yield as well as the viability of the dissociated cells (90% cells were viable). Both normal and mutant chondrocytes formed monolayers (Fig. 1, a and b). Their growth rates were almost similar and nodular assembly of cells was equally recognized on day 4 after plating. But the nodules formed by mutant chondrocytes were much smaller in the average volume of extracellular matrix than those by normal chondrocytes (Fig. 1, a and b).

Immunofluorescence staining of day 7 cultures with antibodies to cartilage PG and type II collagen indicated that in normal culture both cartilage PG and type II collagen localized exclusively in the nodules of normal cultures (Fig. 1, c and e), whereas cartilage PG was completely absent in those of mutant cultures while type II collagen was present (Fig. 1, d and f). These results are consistent with the previous observation (15) that mutant cartilages in vivo are defective in cartilage PG synthesis but have an ability to synthesize type II collagen.

It is noteworthy that the intensity of type II collagen fluorescence was lower and the distribution of the fluorescence was much more uneven in the mutant nodules than in the normal (Fig. 1, e and f). Immunohistochemical detection of type II collagen in tissue sections of normal cartilage usually requires pretreatment of the sections with hyaluronidase to eliminate masking substances (for an example, see reference 15). However, the intensity of type II collagen fluorescence on both cultures of mutant and normal chondrocytes was not augmented by hyaluronidase pretreatment (data not shown). Staining with antibodies to type II collagen of both

samples prepared without air-drying also gave the same fluorescence patterns (data not shown). The results rule out the possibility that the observed differences in staining pattern are due to a denaturation of the antigenic molecules caused by air-drying in the absence of cartilage PG.

In contrast to the apparent differences in the distributions of cartilage PG and type II collagen between normal and mutant cultures as described above, the patterns of link protein were almost similar. The fluorescence appears predominantly in cell surfaces and extracellular matrices of the nodules while weak but significant fluorescence was found in those surrounding the nodules (Fig. 1, g and h). Pretreatment with hyaluronidase gave no difference in the distribution between normal and mutant cultures, but dramatically reduced the staining (data not shown). This suggests that link proteins are bound to hyaluronidase-sensitive materials in both cultures.

Incorporation of Exogenously Added Cartilage PG into Nodules Formed by Mutant Chondrocytes

Mutant chondrocytes were provided with cartilage PG on day 2, by simply adding to the culture medium. Subsequent staining of the cultures with antibodies to cartilage PG demonstrated that the molecule was more incorporated into the extracellular matrices of the nodules as the formation of the nodules progressed and as the concentration increased (Figs. 2 and 3). Continuous culturing until day 7 when the complete nodules were constructed and the addition of 10 µg dry weight of cartilage PG/ml yielded maximum fluorescence. A phase-contrast micrograph (cf. Fig. 6 i) clearly shows that the addition of cartilage PG increased the volume of extracellular matrix in nodules of mutant cultures to the level seen in normal cultures. In contrast, no apparent change in the nodular structure was observed when normal chondrocytes were cultured in the presence of cartilage PG (data not shown).

It is possible that the addition of cartilage PG caused induction of cartilage PG biosynthesis or remodeled proteoglycans in mutant chondrocytes. To investigate this possibility, normal and mutant chondrocyte cultures on day 7 were metabolically labeled with [^{35}S]sulfate with or without addition
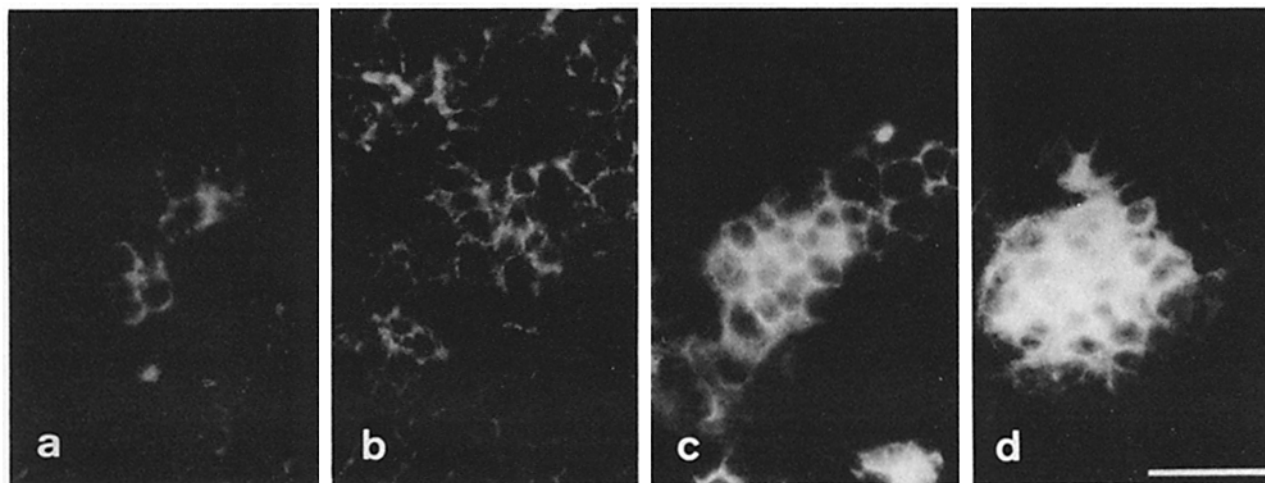

Figure 2. Time course of the incorporation of exogenously added mouse cartilage PG into the mutant chondrocyte culture. Mutant chondrocytes were cultured for 24 h (a), 48 h (b), 96 h (c), and 144 h (d) in the presence of 10 µg of normal mouse cartilage PG/ml. Progressive increase of the fluorescence of cartilage PG with time is apparent. Bar, 100 µm.
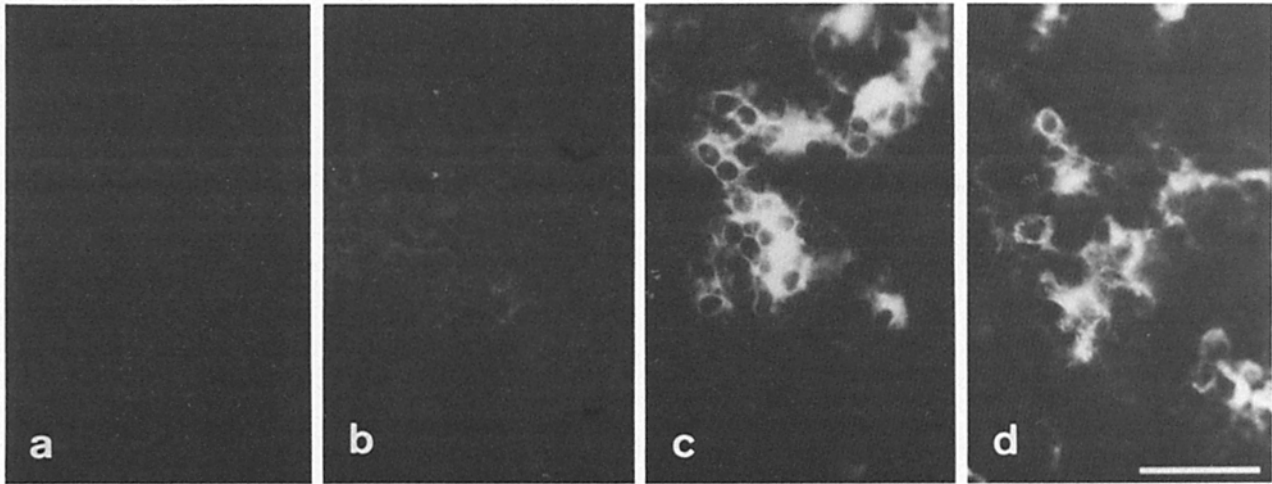
Figure 3. Incorporation of cartilage PG into the extracellular matrix of mutant chondrocytes as a function of the concentration of added cartilage PG. The cells were cultured in the absence (*a*) or presence of 5 μg (*b*), 10 μg (*c*), or 50 μg (*d*) of cartilage PG/ml. After 6 d of incubation, the cultures were stained with antibodies to cartilage PG. The highest intensity of fluorescence is apparent in (*c*). Bar, 100 μm.

of cartilage PG. The cell layers and spent media were analyzed for ^{35}S-labeled proteoglycans by glycerol density gradient centrifugation (Fig. 4). Both the cell layer and medium from the normal showed a typical bimodal sedimentation profile of ^{35}S-labeled proteoglycans of cartilaginous tissues (15). Compared with the normal, all samples from the mutant lacked the faster sedimenting ^{35}S-labeled component that corresponds to cartilage PG (fractions 6–17). Both normal and mutant cultures synthesized and secreted slower sedimenting proteoglycans. This may represent proteoglycan species distinct from cartilage PG in cartilage (so-called small proteoglycans), although their sedimentation rates were slightly faster in the mutant than in the normal. A similar difference in the sedimentation rates of small proteoglycans has also been observed with normal and cmd/cmd cartilages in vivo (15). The addition of cartilage PG to the medium had no effect on the synthesis and secretion of the slower sedimenting proteoglycans. Thus, the appearance of the cartilage PG fluorescence in the mutant nodules resulted from the incorporation of added exogenous cartilage PG.

Response of Mutant Nodules to Exogenously Added Cartilage PG

To investigate whether added exogenously added cartilage PG effects the distribution of type II collagen, antibodies to these molecules were used in double immunofluorescence reaction. The cartilage PG and type II collagen fluorescence had similar localization in nodules of normal day 7 cultures (Fig. 5, *a* and *b*). In contrast, the nodules of mutant chondrocyte cultures without the addition of cartilage PG completely lacked the cartilage PG fluorescence and showed only a weak and unevenly distributed fluorescence of type II collagen (Fig. 5, *d* and *e*). However, the nodules of mutant culture formed with the addition of exogenous cartilage PG were similar to the normal in both intensity and distribution pattern of the cartilage PG and type II collagen fluorescence (Fig. 5, *g* and *h*). Moreover, the addition of cartilage PG increased the volume of extracellular matrix in the nodules nearly to the normal level (Fig. 5 *i*).
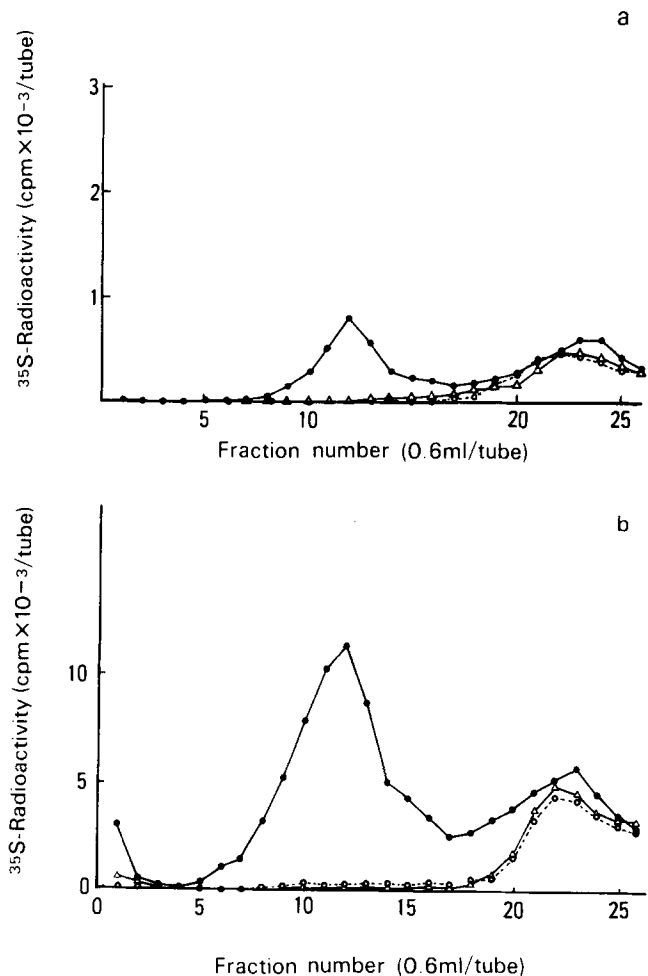

Figure 4. Zone sedimentation profiles of [^{35}S]sulfate-labeled proteoglycans in the cell layers (*a*) and the media (*b*) obtained from normal chondrocyte culture (●) and from mutant chondrocyte cultures in the absence (○) or presence of 10 μg of cartilage-PG/ml (△). Zone sedimentation of the labeled proteoglycans were carried out as described in Materials and Methods. After the centrifugation, tubes were fractionated from the bottom to the top.
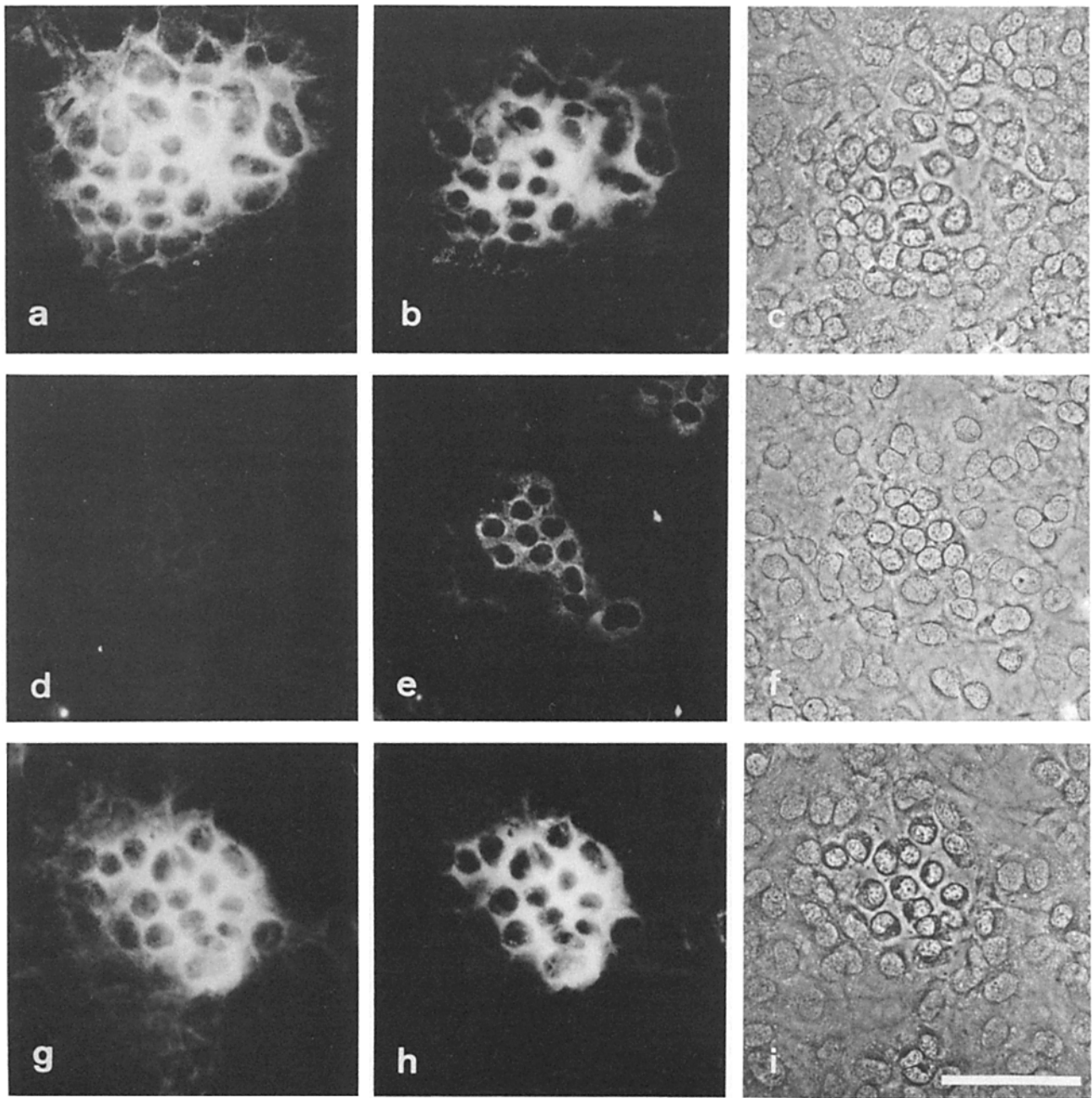
Figure 5. Double immunofluorescence staining of day 7 cultures of normal chondrocytes (*a* and *b*), and of mutant chondrocytes grown in the absence (*d* and *e*) or presence of 10 μg of cartilage PG/ml (*g* and *h*). The cultures were stained simultaneously with rabbit antibodies to cartilage PG (*a, d,* and *g*) and guinea pig antibodies to type II collagen (*b, e,* and *h*). Phase-contrast micrographs of (*a* and *b*), (*d* and *e*), and (*g* and *h*) are shown in *c, f,* and *i,* respectively. Bar, 100 μm.

Inhibition of Cartilage PG Incorporation into Mutant Chondrocyte Nodules by Hyaluronic Acid and Oligosaccharides Derived Therefrom

It is known that hyaluronic acid oligosaccharides or polysaccharides consisting of five or more disaccharide repeating units bind effectively to cartilage PG, thereby competitively inhibiting the interaction of cartilage PG with hyaluronic acid of a high molecular weight (7, 19). Simultaneous addition of hyaluronic acid of high molecular weight (M_r ~8 × 10^5) or hyaluronic acid decasaccharide caused a marked reduction in the incorporation rate of cartilage PG into mutant nodules (Fig. 6, *a* and *c*) with concurrent reduction in

the deposition of type II collagen (Fig. 6, *b* and *d*). In contrast, neither hyaluronic acid octasaccharide nor chondroitin sulfate had such effects (Fig. 6, *e–h*). It appears likely that the interaction of added exogenous cartilage PG with endogenous hyaluronic acid is at least necessary for the incorporation and the correction of the type II collagen distribution.

Structural Specificity of Cartilage PG for the Incorporation into Nodules

Cartilage PG prepared from the Swarm rat chondrosarcoma (22) was incorporated into the nodules of mutant chondrocyte culture as efficiently as mouse cartilage PG (Fig. 7 *c*).
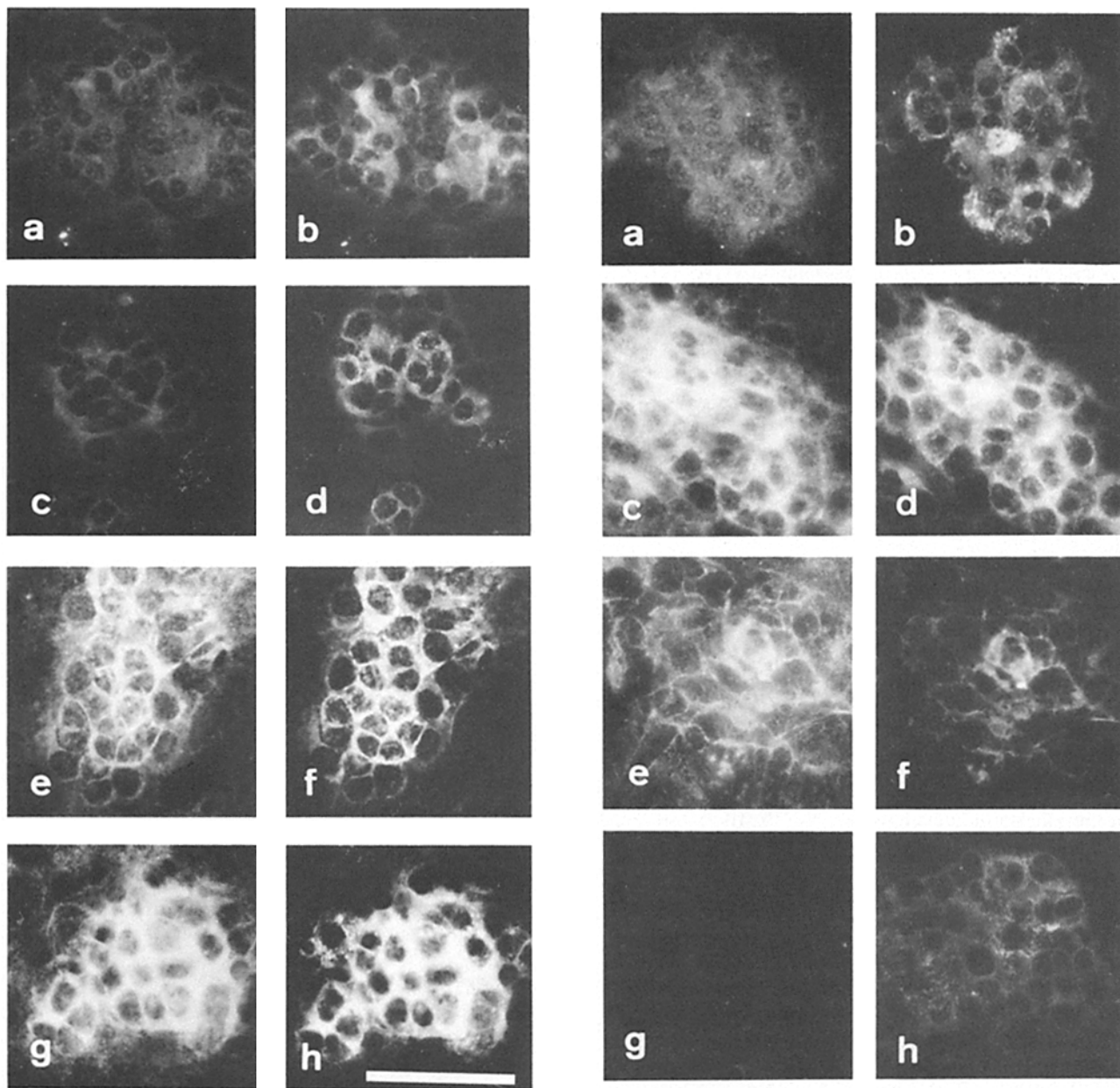
Figure 6. Effects of simultaneous addition of hyaluronic acid or oligosaccharides derived therefrom on the localization of cartilage-PG and type II collagen in mutant chondrocytes culture. The chondrocytes were cultured in media containing 10 µg of cartilage-PG and 50 µg of hyaluronic acid (*a* and *b*), 50 µg of decasaccharide (*c* and *d*), 50 µg of octasaccharide (*e* and *f*), or 50 µg of chondroitin sulfate (*g* and *h*). On day 7, the cultures were stained doubly with rabbit antibodies to cartilage PG (*a, c, e,* and *g*) and guinea pig antibodies to type II collagen (*b, d, f,* and *h*). Bar, 100 µm.

A resultant correction of the nodules with respect to type II collagen distribution pattern was also observed to the same extent as seen with cartilage PG from normal mouse cartilage (Fig. 7 *d*). However, cartilage PG prepared from PG-H (14) incorporated less efficiently than mouse cartilage PG, as assessed by the low intensity of PG-H fluorescence and the incomplete correction of the type II collagen distribution pattern (Fig. 7, *a* and *b*). Proteoheparan sulfate from the mouse Englebreth-Holm-Swarm tumor was neither incorporated
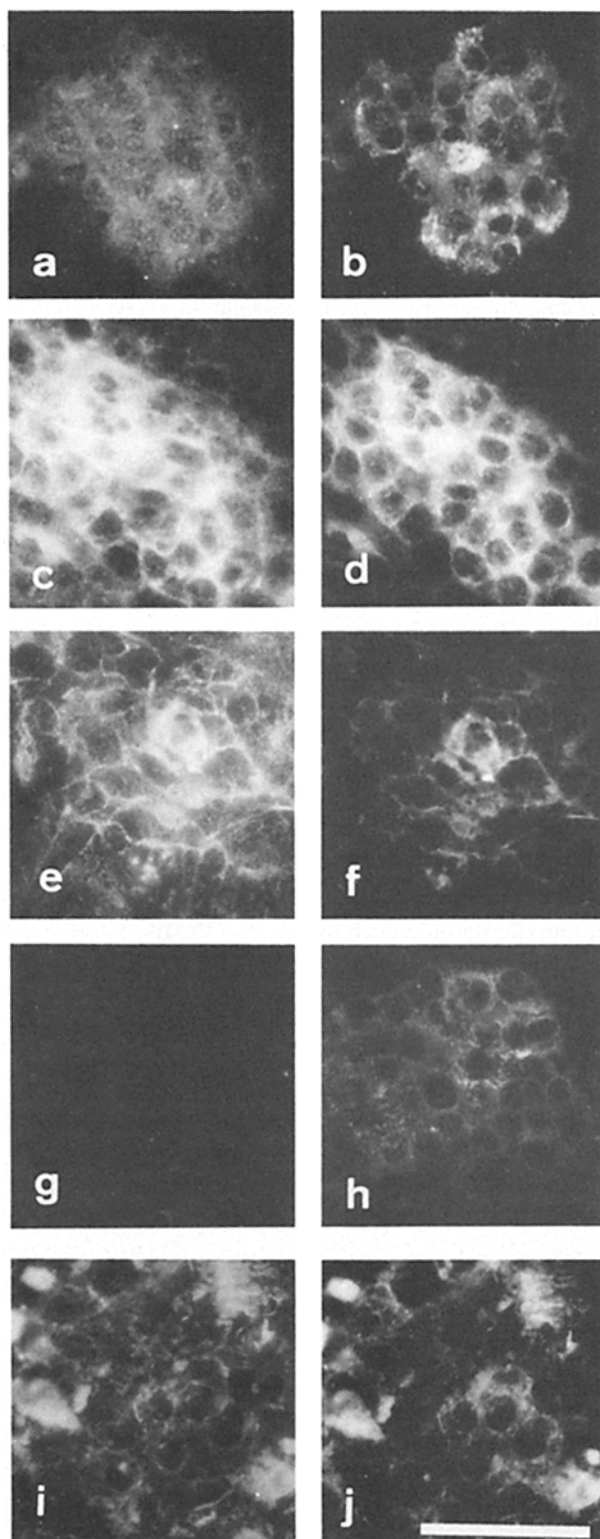

Figure 7. Species specificity of cartilage PG and structural requirements for the incorporation into the mutant nodules. Mutant chondrocytes were cultured for 7 d in the presence of PG-H (50 µg/ml) (*a* and *b*), cartilage PG from Swarm rat chondrosarcoma (10 µg/ml) (*c* and *d*), the hyaluronic acid–binding region (10 µg/ml) (*e* and *f*), the chondroitin sulfate–rich region (10 µg/ml) (*g* and *h*), or the protein-enriched core molecule (10 µg/ml) (*i* and *j*) prepared from rat chondrosarcoma cartilage PG. Cultures were stained simultaneously with guinea pig antibodies to type II collagen (*b, d, f, h,* and *j*), and either rabbit antibodies to PG-H (*a*) or rabbit antibodies to rat chondrosarcoma cartilage PG (*c, e, g,* and *i*). Bar, 100 µm.

into the nodules, nor did it correct the abnormal features of mutant chondrocyte culture (data not shown).[2]

The hyaluronic acid–binding region and chondroitin sulfate–rich region prepared from rat chondrosarcoma cartilage PG by clostripain digestion (4) were examined for their ability to be incorporated into the mutant nodules. The former was incorporated into both the nodules and the surrounding fibroblastic regions with no significant correction of type II collagen distribution pattern (Fig. 7, e and f). The chondroitin sulfate–rich region, in contrast, was neither incorporated into the nodules nor into the surrounding regions, as judged by the complete absence of the cartilage PG fluorescence and by its failure to correct the type II collagen distribution pattern (Fig. 7, g and h). The protein-enriched core molecule prepared from rat chondrosarcoma cartilage PG by chondroitinase AC-II digestion (23) was incorporated into both the nodules and the surrounding regions as was the hyaluronic acid–binding region, but the fluorescence showed a pattern that clots scattered throughout the culture (Fig. 7 i). The fluorescence of type II collagen appeared associated with clots (Fig. 7 j). Because the protein-enriched core molecules have a strong tendency to form aggregates in an aqueous solution, it is likely that the properties caused a nonspecific adsorption of type II collagen released into the medium to the aggregates or coprecipitation of the type II collagen with the aggregates.

Incorporation of the Radiolabeled Cartilage PG into Mutant Chondrocyte Culture and the Effects of Simultaneous Addition of Various Compounds

The immunofluorescence data in Figs. 6 and 7 were obtained with the long-term cultures where the matrix construction appeared to be influenced by many processes, such as degradation. To see whether the cartilage PG fluorescence was a reflection of the incorporation rates of added exogenous cartilage PG or not, the mutant cultures on day 2 were exposed for a short time (24 h) simultaneously to [35S]sulfate-labeled mouse cartilage PG and to various compounds having inhibitory effects on the appearance of the cartilage PG fluorescence in the mutant nodules. When the labeled cartilage PG was added to a final concentration ≤10 μg/ml, ∼1% of the added 35S was recovered in the mutant cell layer, whereas 0.3% or less was in the normal cell layer (data not shown). Because almost all of the bound 35S could be released by treatment with chondroitinase ABC, it is likely that the labeled molecules were located extracellularly in a form bound to the cell surfaces (data not shown). When various compounds were simultaneously added at the same concentrations as those used for the immunofluorescence staining studies, the incorporation of 35S-labeled cartilage PG to the mutant cell layer was inhibited to ∼30% by hyaluronic acid, by decasaccharides derived therefrom, by the hyaluronic acid–binding region of rat chondrosarcoma cartilage PG, or less but significantly, by chick PG-H. In contrast, hyaluronic acid octasaccharides, the chondroitin sulfate–rich region of rat chondrosarcoma cartilage PG, or chondroitin sul-

2. Immunofluorescence staining of normal and mutant chondrocyte cultures revealed the presence of endogenous proteoheparan sulfate in the chondrocyte nodules. Although it was somewhat difficult to determine whether added proteoheparan sulfate was incorporated into the matrices, lack of increased fluorescence suggested that added exogenous proteoheparan sulfate was not incorporated.


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No addition (control) ├──────────────────────────┤(100%)
        + HA        ├──────┤(28%)
        + HA10      ├────────┤(32%)
        + HA8       ├───────────────────────────────┤(106%)
        + HABR      ├─────────┤(30%)
        + CSRR      ├─────────────────────────────┤(95%)
        + Ch4S      ├───────────────────────────────┤(102%)
        + rat PG    ├──────┤(26%)
      + chick PG-H  ├─────────────┤(46%)
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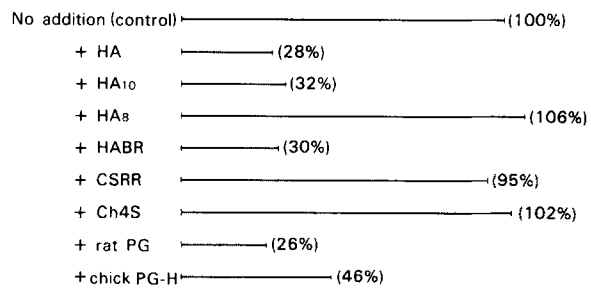
Figure 8. Inhibition by various compounds of the binding of 35S-labeled cartilage PG to mutant chondrocytes in monolayer culture. The compounds tested are; hyaluronic acid (HA), 50 μg/ml; hyaluronic acid decasaccharide (HA10), 50 μg/ml; hyaluronic acid octasaccharide (HA8), 50 μg/ml; the hyaluronic acid–binding region (HABR) from rat chondrosarcoma cartilage PG, 10 μg/ml; the chondroitin sulfate–rich region (CSRR) from rat chondrosarcoma cartilage-PG, 10 μg/ml; chondroitin 4-sulfate (Ch4S), 50 μg/ml; unlabeled rat chondrosarcoma cartilage PG (rat PG), 20 μg/ml; or chick PG-H, 50 μg/ml. 35S-Labeled mouse cartilage PG (10 μg/ml) was added together with the indicated compounds. Values in parenthesis indicate the amounts of radioactivity incorporated to the cell layer during a 24-h incubation. The amount of 35S-labeled mouse cartilage PG incorporation in the absence of inhibitor is designated as 100%.

fate had no such effect (Fig. 8). The results are within those expected from the immunofluorescence staining studies.

Discussion

Previous biochemical studies on the epiphyseal growth plate of cmd/cmd mouse showed that the cartilage synthesized type II collagen and link protein nearly at normal rates, but that the synthesis of the core protein of cartilage PG was undetectable (15, 18). In present reports, we have shown that chondrocytes derived from the mutant cartilage also expressed the metabolic error in culture and yielded nodules where chondrocytes closely packed in greatly reduced extracellular matrix lacking cartilage PG and showing uneven and sparse distribution of type II collagen. We have also shown that these abnormalities were corrected, i.e., changed toward normal, by simply adding to the culture medium cartilage PG isolated from either normal mouse cartilage or Swarm rat chondrosarcoma.[3] The results not only indicate that the abnormalities of the mutant matrix undoubtedly resulted from the deficiency of cartilage PG, but also suggest that in the absence of cartilage PG, type II collagen can hardly be processed to a mature insoluble form or be adequately assembled so that a soluble (immature) form of type II collagen is largely released into the culture medium. It is known that aggregation of the tropocollagen depends upon the presence and amount of sulfated glycosaminoglycans or proteoglycans (20, 21, 27, 32). The present results have for the first time demonstrated that the processing or assembly of type II collagen by growing chondrocytes may be influenced by a failure of the cells to synthesize cartilage PG.

Sommarin and Heinegård (29) showed a time-dependent and saturable binding of [35S]sulfate-labeled cartilage PG to

3. Unlike normal chondrocytes, mutant chondrocytes synthesize and accumulate fibronectin at a high rate. This abnormality can also be corrected by adding the cartilage PG to the culture medium (unpublished observations).

calf articular cartilage chondrocytes in suspension culture. In their study, the added ^{35}S-labeled proteoglycans appeared to be integrated as a part of the pericellular matrix. The results of our immunofluorescence study and binding experiments of radiolabeled cartilage PG also suggest that exogenous cartilage PG was integrated into the matrix of mutant chondrocyte nodules. Hyaluronic acid, decasaccharide derived therefrom, and unlabeled hyaluronic acid–binding region, but not hyaluronate octasaccharide and chondroitin sulfate, inhibited the binding as well as the incorporation. This suggests that the interaction of cartilage PG with hyaluronic acid via the hyaluronic acid–binding region either at the chondrocyte cell surface or in the extracellular matrix of chondrocyte nodules is essential for the binding.

It is known that the interaction of cartilage PG with hyaluronic acid is essentially irreversible in the presence of link protein (6–8). The added exogenous cartilage PG appeared to be assembled into stable hyaluronic acid-link protein–proteoglycan aggregates in the extracellular matrix, since in our immunofluorescence study and binding experiment the fluorescence and radioactivity were detected, respectively, after extensive rinsing in PBS (even using PBS containing 1 mg/ml hyaluronic acid, the same results were obtained). Kimura et al. (19) observed that, when hyaluronic acid of 16 sugar residues, which are long enough to interact reversibly with the cartilage PG even in the presence of link protein, were added to chondrosarcoma cell cultures, the formation of stable aggregates in the medium is retarded but not prevented. In the present study, however, simultaneous addition of cartilage PG and hyaluronic acid decasaccharide to the mutant culture medium disturbed the incorporation into the extracellular matrix of the nodules (Fig. 6). The difference may be due to the possibility that both the amount and length of the decasaccharide were sufficient to displace and release endogenous hyaluronic acid from pericellular matrix to medium, or the excessive amount of the decasaccharide (50 µg/ml) caused the binding of essentially all the added proteoglycan to the decasaccharide.

It is noteworthy that cartilage PG are not incorporated into the surrounding fibroblastic cell zones despite the presence of both hyaluronic acid and link protein (Fig. 1 h). Both the protein-enriched core molecule and the hyaluronic acid–binding region derived from rat chondrosarcoma cartilage PG could be deposited in the nodules of mutant chondrocytes but, unlike the intact proteoglycan, their deposition was not restricted to the chondrocyte nodules but extended to the surrounding fibroblastic zones. Moreover, they had no ability to correct the abnormalities of the mutant chondrocyte nodule. These results suggest that either chondroitin sulfate side chains or chondroitin sulfate–rich regions of cartilage PG is required for both specific incorporation into mutant chondrocyte nodules and correction of the abnormalities of the nodules, although free chondroitin sulfate or the chondroitin sulfate–rich region alone lacks this ability. Chondroitin sulfate side chains have been shown to be essential for the interaction between cartilage PG and chondronectin (11). The observed difference between intact cartilage PG and the hyaluronic acid–binding region (or the protein-enriched core molecule) derived therefrom suggests the participation in the process of cartilage matrix assembly of such a molecule as one displaying affinity for chondroitin sulfate.

Cartilage PG isolated from chick embryo cartilage (PG-H)

was neither incorporated into the matrix nor corrected the abnormalities of mutant chondrocyte nodules as well as the mammalian proteoglycans. PG-H differs from the chondrosarcoma cartilage PG in that it contains keratan sulfate side chains (22, 23), is rich in high mannose-typed N-linked oligosaccharides (30), and has a homologous, but partially different, core polypeptide (unpublished observations). It may be that some of these species-specific structural factors interfere with proteoglycan-link protein interaction involved in the formation of stable proteoglycan aggregates. Alternatively, species-different environments might expose PG-H more to the degradation processes so as to reduce its ability to interact with hyaluronic acid.

We believe that cmd/cmd chondrocyte cultures are well suited for determination of the mechanism of matrix assembly, because the cells yield nodules with a deficient matrix that can be modulated by exogenous substances with well defined chemical structure.

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