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Chondrodysplasia
collagen
growth cartilage
Kniest's disease
proteoglycans
pseudoachondroplasia

Gel Electrophoretic Studies on Proteoglycans and Collagen of Abnormal Human Growth Cartilage: Proteoglycan Abnormalities in Pseudoachondroplasia and in Kniest's Disease

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Extract

The microchemical study of growth cartilage biopsies may improve the classification and the genetic advice of some types of growth disturbances and contribute to the understanding of biochemical defects.

Small tibial growth cartilage biopsies were performed during orthopedic surgery in cases with achondroplasia, pseudoachondroplasia (three cases), Kniest's disease (two cases), diastrophic dwarfism (two cases), parastrematic dwarfism, pycnodysostosis, mucolipidosis type III, Blount's disease, and in three normal growing children. Five human fetal cartilages were also studied.

The proteoglycans were extracted with 4 M guanidinium chloride. After dialysis against 8 M urea at pH 7, the proteoglycans were obtained by ion chromatography in urea on DEAE-cellulose and submitted to gel electrophoresis on polyacrylamide-agarose gels. The gel electrophoresis of the proteoglycans of growth cartilage of normal growing children gave two metachromatic bands situated close one to another. The proteoglycans extracted from fetal growth cartilage gave a single band with a slightly slower migration. An abnormal gel electrophoretic pattern was found in pseudoachondroplasia and in Kniest's disease. In pseudoachondroplasia a single wide band was found; in overcharged tubes several thin, more rapid bands appeared in addition to the main band. In Kniest's disease three bands were found.

In all of the other syndromes studied two normally or almost normally situated bands were present. Small differences in the width and intensity of the bands observed in several cases were difficult to assess. In all cases except mucolipidosis III and Kniest's disease the collagen was extracted by using limited cleavage and solubilization with pepsin, purified and analyzed by polyacrylamide gel electrophoresis. A type of collagen with a single α band was found.

Speculation

Pseudoachondroplasia and Kniest's disease are due to genetic alterations of proteoglycans. These abnormalities produce the growth disturbance and the skeletal dysplasia. It is likely that the alteration is limited to cartilage or to cartilage and bone. The abnormal endoplasmic reticulum found in both diseases suggests defects in synthesis and/or transfer of proteoglycans or of their precursors. However, the possibility of a different primary defect affecting secondarily the gel electrophoretic pattern of extracted proteoglycans could not be excluded.

The histochemical and microchemical study of growth cartilage biopsies may improve the classification and the genetic advice of some types of growth disturbances. The data obtained might also contribute to the understanding of the pathogenesis of the syndromes investigated. In previous studies we found abnormalities of glycosaminoglycans and collagen in some types of abnormal human growth cartilage (11, 14). In the present study a gel electrophoretic analysis of proteoglycans and collagen extracted from normal and abnormal human growth plate biopsies was performed.

MATERIAL AND METHODS

CASE MATERIAL

The proximal tibial growth cartilage of 13 cases with growth disturbances was studied (Table 1). Three normal growing children aged 5, 7, and 12 years were studied as control subjects. Five fetal specimens selected from cases of therapeutic abortion and obtained within 10 min of the abortion were also studied. The gestional age of the fetuses was determined from the crown-rump length.

BIOPSY PROCEDURE

In all the cases (except the fetuses) the cartilage was obtained by biopsy performed during orthopedic surgery, according to a previously described procedure (11).

TISSUE PREPARATION

The biopsy fragments and the fetal specimens were quickly frozen with carbon dioxide and stored at −60°. Undecalcified frozen sections were cut in a cryostat at −20°. Groups of 40 μm thick sections for microchemical determinations were alternated with groups of sections 14 μm thick to be used for histochemical staining. The 40-μm thick sections were freeze-dried at −40° for 4–5 days at a vacuum of about 0.01 mm Hg. The cartilage was separated from bone by microdissection of the freeze-dried sections under a binocular dissecting microscope.

GEL ELECTROPHORETIC ANALYSIS OF PROTEOGLYCANS

The microdissected lyophilized sections were extracted with 4 M guanidinium chloride, 0.05 Tris-HCl, pH 7.2, at 4° with magnetic stirring for 48 hr (10).

After dialysis against 8 M urea in Tris-HCl buffer, 0.05 M, pH 7.0, at 4° (Dia-Flo pressure cell, Amicon, UM 20E membrane) the proteoglycans were obtained by ion chromatography on a DEAE-cellulose column in the 2 M NaCl fraction (1, 2). This fraction was dialyzed against the 8 M urea in Tris-HCl buffer, pH 7.0 solution, to decrease the NaCl concentration to 0.02 M. The proteoglycans were subjected to gel electrophoresis on large pore composite polyacrylamide-agarose gels. The method of McDevitt and Muir (5), modified slightly (1.2% acrylamide and 0.7% agarose), was used. Samples of proteoglycans equivalent to 1.5–2.5 μg hexuronic acid were layered on each tube. The electrophoresis was performed in a cold room with a current of 5 ma/tube and a voltage gradient of about 20 V/cm, the duration of the run being about 60 min. The gels were stained with 0.2% toluidine blue in 0.1 N acetic acid and the excess dye was removed with 0.1 N acetic

Table 1. *Study of proximal tibial growth cartilage*

Case	Age. yr
Pseudoachondroplasia	11 7/12
Pseudoachondroplasia	7 6/12
Pseudoachondroplasia	7
Kniest's disease	8
Kniest's disease	10
Achondroplasia	11
Diastrophic dwarfism	8
Diastrophic dwarfism	10
Blount's disease	13
Pycnodysostosis	13
Parastrematic dwarfism	9
Mucolipidosis type III	14
Multiple exostoses	10
Normal	5
Normal	7
Normal	12
Fetuses (5 cases)	15–19[1]

[1] Weeks of gestational age.

acid, followed by washings of water. A sample of chondroitin sulfate was included in all runs as a reference standard. Two tubes of each sample were analyzed and runs were repeated.

GEL ELECTROPHORETIC ANALYSIS OF COLLAGEN

After the extraction of the major part of proteoglycans the cartilage was dissolved by using the limited cleavage and solubilization with pepsin technique according to the method of Miller (6). After incubation the digestion mixture was clarified by centrifugation and the collagen was precipitated by addition of sodium chloride. The precipitate was retrieved by centrifugation, redissolved in 1 M sodium chloride, pH 7.5, and dialyzed against a large volume of 0.05 M acetic acid. Further purification was performed using the method of Miller *et al.* (7). The material obtained was analyzed by polyacrylamide gel electrophoresis according to Stark and Kühn (15). Samples of collagen extracted and purified by the same method from the skin and cartilage of a baboon were included as controls. Acid-soluble collagen from rat tail tendon was also analyzed in the same run.

RESULTS

PROTEOGLYCANS

The gel electrophoresis of the proteoglycans of growth cartilage of normal growing children yielded two metachromatic bands situated close one to another. The proteoglycans extracted from fetal growth cartilage gave a single band with a slightly slower migration (Fig. 1). The chondroitin sulfate standard gave a single band which migrates faster than the proteoglycans.

An abnormal gel electrophoretic pattern of proteoglycans was found in pseudoachondroplasia and in Kniest's disease.

In psuedoachondroplasia a single wide band was found (Fig. 2). In overcharged tubes several thin and more rapid bands appear in addition to the main wide band (Fig. 3). The pattern is different both from that found in normal children (two bands) and in fetuses (one narrow and slightly delayed band).

In Kniest's disease three bands were found. The third band is faster than the normal bands. The same pattern was found in the two cases studied (Fig. 4).

In all the other syndromes studied two normally or almost normally situated bands were found. Small differences in the wideness and the intensity of the bands observed in several cases are difficult to assess.

COLLAGEN

The gel electrophoretic analysis of collagen was performed in the normal children and in all of the pathologic cases except the cases with mucolipidosis III and Kniest's disease.

The collagen extracted from the normal children and from the cartilage of the baboon gave a single α-chain band. All the abnormal growth cartilages studied gave a single α band with the same migration as the band of normal cartilage. In contrast the collagen extracted from the baboon skin and the acid-soluble collagen from the rat tail tendon yielded two α-chain bands (Fig. 5).

DISCUSSION

With the method used the proteoglycans were obtained in highly "dissociative" conditions and no stained material remained at the gel origin. The method of extraction and purification we used in this study yields cartilage proteoglycans with very similar properties (by gel chromatography, distribution in a density gradient, and composition) to the "subunits" (A1-D1) isolated by the Hascall and Sajdera method (1). However, the existence of dimers or small aggregates (9) could not be excluded. Addition of proteolysis inhibitors does not alter electrophoretic results (13).

The present study demonstrates a definite abnormal gel electrophoretic pattern of "dissociated" proteoglycans obtained from the growth cartilage in two types of chondrodystrophic dwarfs: pseudoachondroplasia and Kniest's disease. This suggests strongly that the genetic defect in these diseases alters the molecular populations of proteoglycans of the growth cartilage. Clinical data suggest that the alteration might be limited to the chondrocytes or to chondrocytes and bone cells. Other connective tissues are probably not or less affected. Further studies are, however, necessary to elucidate this point. The possibility of a different primary defect affecting secondarily the gel electrophoretic pattern of extracted proteoglycans could not be excluded.

Both pseudoachondroplasia and Kniest's disease are spondyloepiphyseal dysplasias with important alterations of the epiphysis and spine, and with severe dwarfism. In pseudoachondroplasia the chondrocytes contain many large vacuoles bound by the endoplasmic reticulum with alternately electron-dense and electron-lucent layers. The vacuoles were observed by Cooper *et al.* (3) and by us in two of the three cases included in this study in which an ultrastructural examination was performed. In two cases of Kniest's disease included in the present study the ultrastructural investigation disclosed a dilated rough surfaced endoplasmic reticulum cisternae containing a granulo-fibrillar material (13).

In our cases microscopic optical studies on semithin Epon sections and on frozen sections showed that the inclusions of pseudoachondroplasia were positive for protein staining and not metachromatic, whereas the inclusions in Kniest's disease contained metachromatic material (13).

The relationship between the abnormal appearance of the endoplasmic reticulum and the abnormal pattern of the cartilage proteoglycans is still unknown. The abnormal endoplasmic reticulum suggests that the abnormalities of proteoglycans are due to troubles in the synthesis and/or transfer of these compounds or of a factor which plays a role in the formation of small aggregates. The intracellular accumulation of some precursor(s) of a proteoglycan population is one of the possibilities to be considered in pseudoachondroplasia.

It is known that in pseudoachondroplasia the clinical and roentgenologic alterations are not present at birth but become evident during the first or second year of life. This might suggest that the genetic defect does not alter the fetal proteoglycans. It was shown that the gel electrophoretic pattern of growth cartilage proteoglycans is different in fetuses from that found in older animals (12 and the present study). In humans, the change takes place in the first months of extrauterine life (13). An alternative

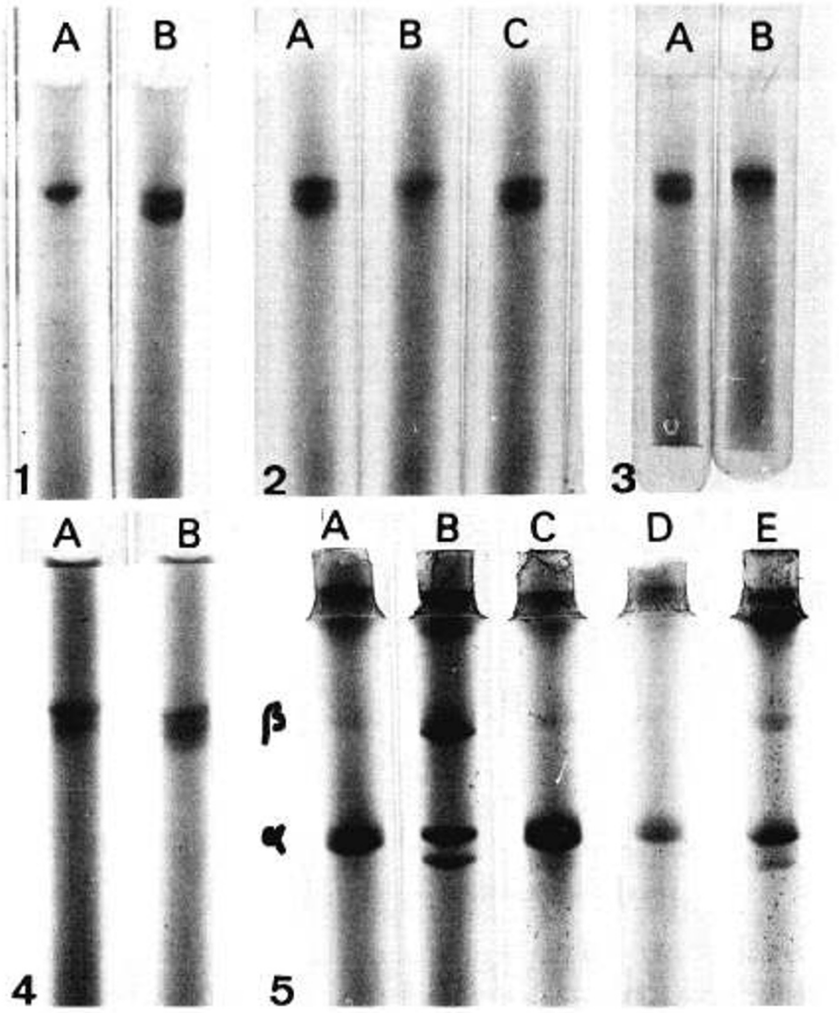

Fig. 1. Proteoglycans. *A:* fetal proteoglycans; *B:* Proteoglycans of a 7-year-old child.
Fig. 2. *A:* normal; *B:* pseudoachondroplasia; *C:* diastrophic dwarfism.
Fig. 3. *A:* normal; *B:* pseudoachondroplasia, overcharged tube.
Fig. 4. *A:* normal; *B:* Kniest's disease.
Fig. 5. Collagen. *A:* pseudoachondroplasia; *B:* acid-soluble collagen, rat tendon; *C:* baboon cartilage; *D:* diastrophis dwarfism; *E:* baboon skin.

hypothesis might suggest that the abnormalities become apparent when the child walks and the abnormal cartilage is under stress (3).

Some subtle differences were observed in the gel electrophoretic pattern of proteoglycans in several other syndromes (*e.g.*, differences in the intensity and the width of the normal situated bands or small differences in migration). However, these differences are difficult to assess and the study of additional cases is necessary. On the other hand, identity of position of two bands in our system does not mean necessarily identity of the proteoglycan populations.

The analysis of the small amount of collagen extracted from biopsies of normal and abnormal growth cartilage had a limited purpose. We intended to find out whether abnormal growth cartilage contained collagen displaying two types of chains instead of one. It was found from the literature that in certain circumstances (tissue culture, arthrosic cartilage, preincubation with lysosomal enzymes) (4, 8), the chondrocytes produce a significant amount of bone and fibrocyte type of collagen $[\alpha_{1(I)}]_2\alpha_2$ in addition or instead of the cartilage type of collagen $[\alpha_{1(II)}]_3$. In previous histochemical studies we found abnormal fibrosis in the matrix of some pathologic growth cartilages (11, 14), and we made the suggestion that the chondrocytes may have an incompletely or abnormal differentiation. However, in all the pathologic cases included in this study, a single α band was found. However, with the method used small amounts of fibrocytic type of collagen might be undetected.

SUMMARY

The gel electrophoretic pattern of proteoglycans extracted from small biopsies of tibial growing cartilage was studied in cases with achondroplasia, pseudoachondroplasia (three cases), Kniest's disease (two cases), diastrophic dwarfism (two cases), parastrematic dwarfism, pycnodysostosis, mucolipidosis type III, Blount's disease, and multiple exostoses. The results were compared with those obtained in three normal growing children of similar age and in five human fetuses. Gel electrophoresis of proteoglycans showed abnormal patterns in the cases with pseudoachondroplasia and with Kniest's disease. In the same cases, except mucolipidosis III and Kniest's disease, the collagen was analyzed by polyacrylamide gel electrophoresis. In all the cases studied, a cartilage type of collagen with a single α band was found.

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Glycine
glycine synthase system

ketotic hyperglycinemia
vitamin B_{12} deficiency

The Vitamin B_{12}-deficient Rat as a Possible Model of Ketotic Hyperglycinemia

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Extract

The rate of oxidation to respiratory CO_2 of both carbon 1 of propionate and carbon 1 of glycine was decreased significantly in vitamin B_{12}-deficient rats, to 50% and 82% of the control rate, respectively. The activity of the glycine synthase system was reduced during vitamin B_{12} deficiency to 25% of control activity. Serine hydroxymethyltransferase activity was similar for vitamin B_{12}-deficient and control rats. Plasma glycine concentration in vitamin B_{12}-deficient rats (253 ± 16 nmol/ml) did not differ significantly from that of control rats (226 ± 12 nmol/ml). Propionate oxidation was significantly impaired in biotin-deficient rats. However, this impairment, to 66% of the control rate, was not as large as that generated by vitamin B_{12} deficiency. In contrast to the result obtained in vitamin B_{12}-deficient animals, no significant decrease in glycine oxidation could be demonstrated in biotin-deficient animals Plasma glycine concentration of fasted biotin-deficient rats (339 ± 26 nmol/ml) did not differ significantly from that of their controls (371 ± 32 nmol/ml).

Speculation

Activity of the glycine synthase system is reduced in both the ketotic and nonketotic forms of hyperglycinemia. The decrease in glycine synthase system activity in vitamin B_{12}-deficient rats may be generated by a mechanism similar to that in ketotic hyperglycinemia, and therefore vitamin B_{12}-deficient rats may be useful to study this mechanism.

In 1961, Childs *et al.* (5) described a patient with a disorder with episodic vomiting, lethargy, ketosis, developmental retardation, and hyperglycinemia and hyperglycinuria. Later studies demonstrated that this disease resulted from an inherited deficiency of propionyl-CoA carboxylase (ATP hydrolyzing) (EC. 6.4.1.3) (13). Similar symptoms may be present in patients with a deficiency in methylmalonyl-CoA mutase (EC. 5.4.99.2) (17, 19) and presumed β-ketothiolase deficiency (acyl-CoA:acetyl-CoA C-acyl transferase, EC. 2.3.1.16) (9). The role of these enzymes in propionate metabolism is shown in Figure 1.

The defect in glycine metabolism in the different forms of ketotic hyperglycinemia appears to be secondary to the impairment in organic acid metabolism. The mechanism of interaction of organic acid metabolism with glycine metabolism is as yet unexplained. The major pathway for glycine degradation in mammals appears to be via conversion to methylenetetrahydrofolate and CO_2, catalyzed by the glycine synthase system (EC. 2.1.2.10) (28). The ketotic hyperglycinemia syndrome differs clinically from the metabolic disease, nonketotic hyperglycinemia, a disorder first described by Gerritsen *et al.* (7). Nonketotic hyperglycinemia is characterized principally by severe mental retardation and caused by an inherited primary defect in the glycine synthase system (26).