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MATRIX COMPOSITION OF CARTILAGINOUS ANLAGEN IN ACHONDROGENESIS TYPE II (LANGER-SALDINO)

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TABLE OF CONTENTS

1. ABSTRACT

Skeletal dysplasias represent *in vivo* models of genetic defects. Achondrogenesis type II (Langer-Saldino), caused by a genetic defect in the major cartilage matrix protein, collagen type II, is a rare and severe skeletal dysplasia. It comprises a severe derangement of the fetal growth plate cartilage with subsequent ossification defects. In this study, we analyzed the matrix composition and cell differentiation pattern in 3 relatives with achondrogenesis type II. Most strikingly we found a strongly reduced collagen type II and moderately reduced aggrecan proteoglycan content in the dysplastic cartilage matrix. Type II collagen is, at least to some extent, replaced by collagens type I III, and VI. Ultrastructural analysis of the dysplastic cartilage matrix demonstrated a distended rER (rough endoplasmic reticulum), which is typical for this condition and most likely related to improper processing and retention of genetically altered type II collagen. Immunostaining for type IIA and X collagens suggest a severe delay in chondrocyte maturation.

Thus, the genetic defect in the present cases leads most likely to a severe retention of collagen type II in the rER and, therefore, a strongly reduced collagen deposition and replacement by other interstitial collagens. However, the latter are less efficient in binding aggrecan proteoglycans in the dysplastic cartilage matrix. Additionally, a delay in chondrocyte maturation appears to be important in achondrogenesis type II.

2. INTRODUCTION

Achondrogenesis type II (Langer-Saldino) is a rare and severe skeletal dysplasia with a clinical picture very similar to the type I subforms of achondrogenesis (1, 2). Both types belong to the short-trunk osteochondrodysplasias. In achondrogenesis type II the limbs are considerably shortened. Achondrogenesis type II is a lethal disorder, pre- or immediately postnatally at latest. The major radiographic signs are markedly deficient ossification of the vertebral bodies and absence of pubic and ischial ossification (2). Histopathologically, deficiency of cartilage matrix and enlargement of the chondrocytes is typical. There are increased and broadened cartilage channels with marked peri-chanalicular fibrosis. Growth plate architecture is severely deranged. Earlier investigations suggested an autosomal recessive disorder (3, 4), more recent studies have favored autosomal dominant inheritance (5-7).

Nowadays, many skeletal dysplasias are rather easily identifiable at an early stage of gestation limiting their clinical impact in practice. Still, skeletal dysplasias are of high scientific significance as they represent *in vivo* model systems in the human in order to understand the implications of genetic defects. Previous studies identified

Table 1. Antibodies and pretreatments used for immunohistochemistry

Antigen	Type	Dilution	Digestion	Source
Vimentin	m	1:200	-	Dako (Denmark)
S-100	r	1:10000	P	Dako (Denmark)
collagen I	r	1:200	H, Pt	Synbio (FRG)
collagen II – E8	m	1:50	H, P	Holmdahl (39)
collagen II – D3	m	1:20	H, P	Holmdahl (39)
Collagen II – C1	m	1:50	H, P	Holmdahl (39)
Collagen II	r	1:50	H, P	Novacastra (United Kingdom)
Collagen IIA	r	1:1000	H, Pt	Oganesian (20)
Collagen III	r	1:1000	H, P	Dr. Günzler (Aventis Pharma, FRG)
Collagen VI	r	1:2000	H, Pt	Dr. R. Timpl (München, FRG)
Collagen X	m	1:50	H, Pt	Girkontaite (40)

Primary antibodies and enzymatic pretreatments used for immunohistochemical analyses (m: mouse monoclonal; r: rabbit polyclonal; H: hyaluronidase (2 mg/ml, phosphate buffered saline (PBS), pH 5, 60 min at 37°C); P: pronase (2 mg/ml, PBS, pH 7,3, 60 min at 37°C); Pt: protease XXIV (0,02 mg/ml, PBS, pH 7,3, 60 min at RT)

collagen type II as basic defect of achondrogenesis type II (6, 8), which is the main matrix constituent of fetal hyaline cartilage. However, it is still not known, whether and how other collagens replace the deficient type II collagen. Type I collagen was identified in the fetal growth plate (6, 9-11), but it remains unclear whether type I collagen is derived mainly from the perivascular fibrotic spaces (12) or replaces type II collagen within the cartilage matrix. No information exists on the distribution of other collagens such as type III collagen, another interstitial collagen, and type VI collagen, which is able to form a fine-fibrillar network un-related to the other interstitial collagens. Type VI collagen is found mainly in the pericellular matrix in hyaline cartilage (13, 14). Another unresolved question is whether the differentiation cascade typical for physiological fetal growth plate is preserved within achondrogenesis type II (11). These questions were addressed in this study using monospecific antibodies for major matrix components and markers of chondrocytic differentiation and maturation. We studied 3 cases of achondrogenesis type II (Langer-Saldino) in one family together with 7 skeletally normal cases.

3. METHODS

3.1. Tissue preparation

Growth plate specimens (knee and hip joints) from three affected and one unaffected sib-fetus with non-consanguineous parents and from 6 skeletally normal unrelated fetus were fixed in 10% formalin, decalcified in 0.3 M EDTA (pH 7.5), dehydrated, cleared in xylene, and embedded in paraffin. Three to 5 µm sections were cut and stored at room temperature until use and stained with haematoxylin-eosin.

3.2. Histochemical Methods

Mucopolysaccharides: The *cartilage typical glycosaminoglycans (GAGs)*, which are found abundantly in cartilaginous tissues, were visualized by toluidine blue staining (10 min, 0.3% toluidine blue (Merck, FRG); pH 3.65, room temperature).

Collagens: The presence of collagens in the extracellular tumor matrix was demonstrated by Masson-Goldner staining.

3.3. Transmission electron microscopy

Conventional transmission electron microscopy was performed according to Spurr (15), modified by Schulz (16). Briefly, small cartilage pieces were fixed with glutaraldehyde-formol over night (1.25% glutaraldehyde, 2% formalin) and cleared in phosphate-buffer (18.76 mg/ml NH_2PO_4, 4.28 mg/ml NaOH, pH7,5). After postfixation with 1% OsO_4 for 2h and re-washing in the phosphate-buffer the samples were dehydrated in an acetone-series. Then, samples were infiltrated with an epoxy resin-acetone series (50% for 30 min, 75% over night, two times 100% for 2 hours) and embedded in epoxy resin according to Spurr and Schulz in standard gelatin capsules (15;17). The epoxy resin was polymerized over night at 70°C. For light microscopical control, semi-thin sections (0.5-1µm) were made and stained with methylen-blue and azure II (18). For electron microscopical analysis ultra-thin sections (50-100 nm) were placed on Formvar laminated copper grids and stained with uranyl-acetate and "lead-citrate".

3.4. Immunohistochemistry

Deparaffinized sections were enzymatically pretreated (table 1), incubated with primary antibodies (table 1) overnight at 4°C and visualized using alkaline-phosphatase-labeled secondary antibodies and 3-hydroxy-2-naphtylacid 2,4-dimethylanilid as color substrate (19). Nuclei were counterstained with haematoxylin.

4. RESULTS

4.1. Clinical data, macroscopy, and radiology

We examined four fetuses conceived by the same non-consanguineous couple miscarried in the 15^{th}, 17^{th} and 20^{th} week of gestation. Three of them were male, one female. Three of them showed micromelic dwarfism, hydrops, prominent forehead, flat face and micrognathia, and cervical hygroma. Radiographs showed (figure 1b) enlarged calvaria, deficient mineralization of vertebral bodies, lack of ossification of sacral, ischial and pubic bones, flared and cupped metaphyses, short tubular bones, and horizontal short ribs without fractures and pulmonary

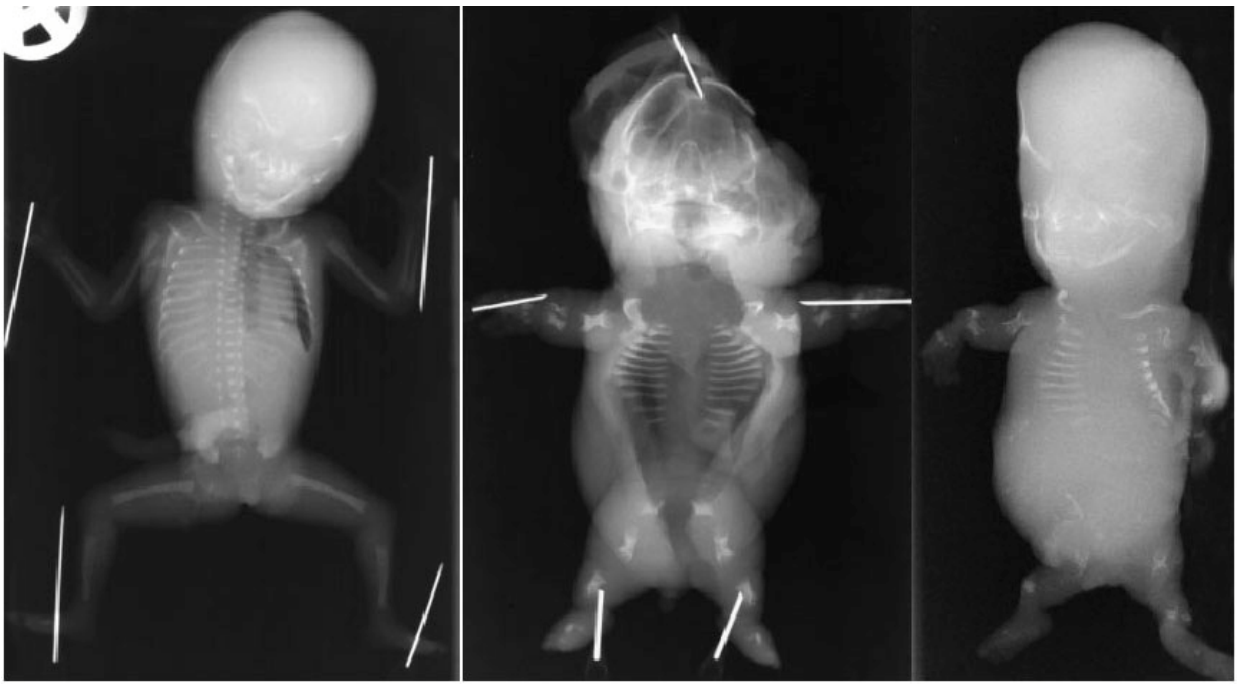

Figure 1. a) Normal radiograph of the healthy fetus (17th week of gestation). b) Radiograph from one of the affected fetus with characteristics of achondrogenesis type II (20th week of gestation): enlarged calvaria with normal ossification, absent mineralization of vertebral bodies, lack of ossification in the os sacrum, the ischial and pubical bones, horizontal and shortened ribs without fractures, normal clavicles, flared, cupped metaphysis, short tubular bones, lack of ossification of the talus and calcaneus.

hypoplasia. One fetus also had a cleft palate. Internal organs were normal. The male fetus manifested no external or internal nor radiographic abnormalities (figure 1a); his loss at 17 weeks of gestation was due to cervical insufficiency, ascending infection and retroplacental hematoma.

4.2. Histology

Histological examination (figure 2a, b) showed derangement of the growth plate cartilage without abnormalities in the surrounding soft tissues. The growth plate cartilage lacked a structured proliferative zone with typical cellular columns. Numerous vascular channels were observed throughout the cartilage. On the level of cellular enlargement chondrocyte hypertrophy did not seem to be reduced. In bone, the major abnormality appeared to be large residual cartilage remnants in somewhat irregular bone trabeculae; osteoblasts and osteocytes did not appear to be significantly altered. All the other connective tissues (tendons, joint capsule) appeared to be not affected.

4.3. Expression of S-100 protein as marker of chondrocytic differentiation

S-100 was found within chondrocytes (figure 2f) as in physiological growth plate cartilage and surrounding neural tissue, but not in osteoblasts or any other connective tissue. Vimentin, a marker of mesenchymal differentiation, was also demonstrable in all chondrocytes (figure 2e) and other connective tissue cells.

4.4. Histochemical detection of mucopolysaccharides and collagens

Histochemically, the most striking aspects of the growth plates of all 3 abnormal fetuses was a strongly reduced amounts of collagen (figure 2c, g, h) and moderately reduced proteoglycan (figure 2d) content throughout the growth plate cartilage compared to the unaffected one (and skeletally normal fetus processed in parallel). In contrast, the bone matrix (figure 2i), the surrounding fibrous matrix, and also the mesenchymal stroma of the vascular channels within the fetal cartilage showed normal amounts of collagen (and – as expected - the absence of GAG staining).

4.5. Immunocolocalization of collagen type II and aggrecan

Immunolocalization of the main cartilage components, collagen type II (figure 3c-f) and aggrecan proteoglycan (figure 3 a, b), confirmed the strongly reduced presence of collagen type II molecules within the cartilage matrix. Very interestingly, several of the monoclonal antibodies used in our study failed to show significant staining signals (figure 3f), whereas others showed a severely reduced staining compared to normal (figure 3d, e). As expected, the cartilage remnants within the bone showed the same staining pattern found in the growth plate cartilage. Outside the cartilage and bone no staining for collagen type II and aggrecan was observed (both, in the diseased and normal specimens).

4.6. Immunolocalization of phenotypic marker collagen types IIA and X

Immunostaining for type IIA collagen showed the presence of this particular splice variant in all cartilage zones including the cartilage remnants within the bone trabeculae (figure 3g). Collagen type IIA was not concentrated within the epi-/peri-chondral area as in the

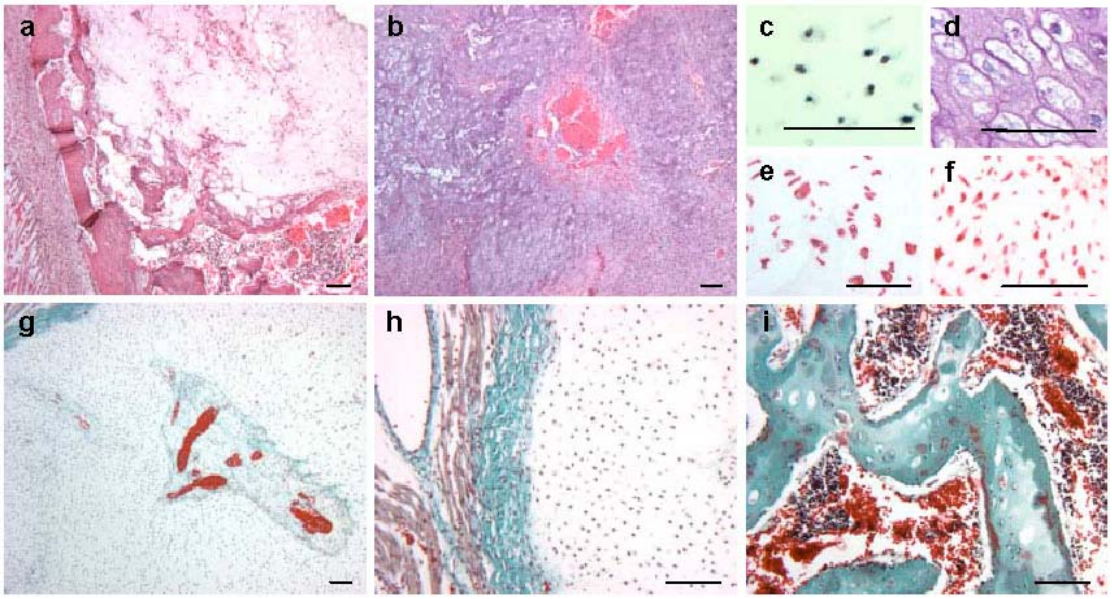

Figure 2. Conventional histological (HE) staining of the fetal growth plate shows the severely deranged architecture (a, b). Histochemical analysis documents the reduced content of collagens in the cartilage matrix (g,h, detail: c), but not bone (i) and the surrounding tissue (h). Also the proteoglycan concentration appears to be reduced (d). Immunostaining for vimentin (e) and S-100 protein (f) demonstrates the positivity of both cytoproteins as expected for chondrocytic cells. (magnification bars: 100 µm).

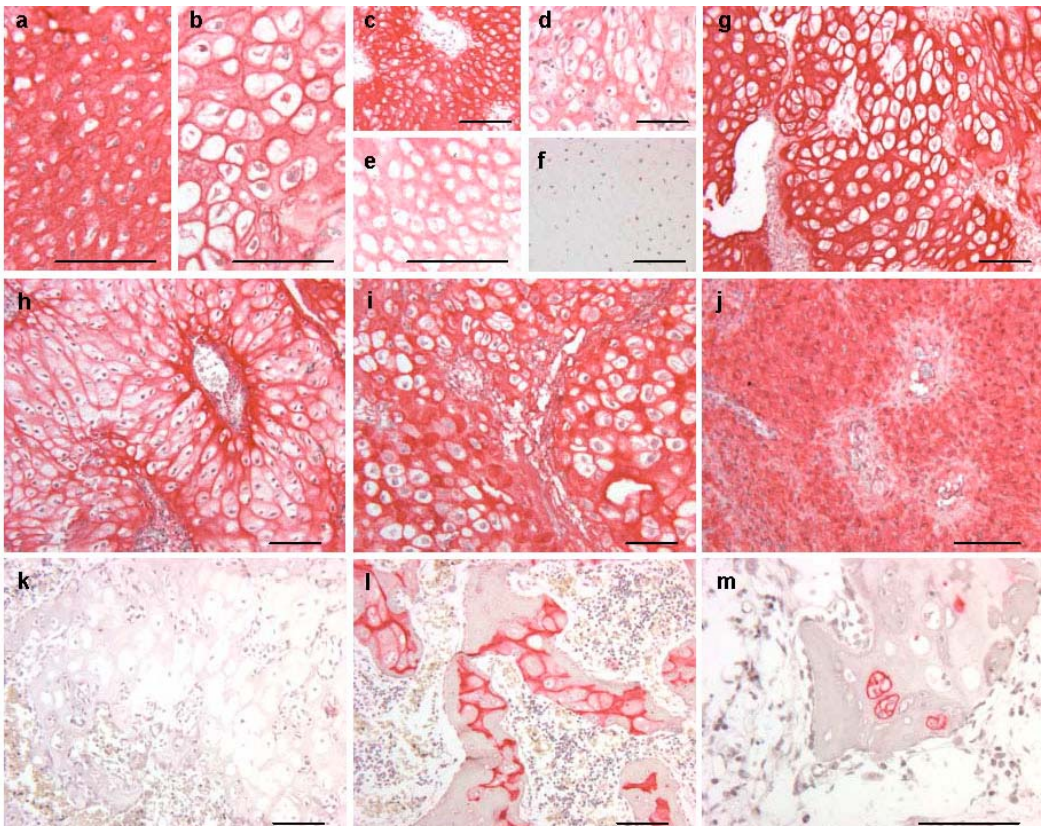

Figure 3. Immunohistochemical analysis of the cartilage matrix in achondrogenesis type II shows a moderate positivity for aggrecan proteoglycan (a,b) and the presence of type II collagen with some (c), but only weak or no staining with other monoclonal antibodies (d-f). A strong staining was observed with a polyclonal antibody to type II A collagen (g). Type I collagen was found concentrated round the vascular channels, but also within the cartilage matrix (h). Type III (i) and VI (j) collagens were observed throughout the cartilage matrix. No type X collagen could be found in the cartilage zone next to the area of bone formation (k), but appeared in the cartilage cores within the newly formed bone trabecules (i, m). (magnification bars: 100 µm).

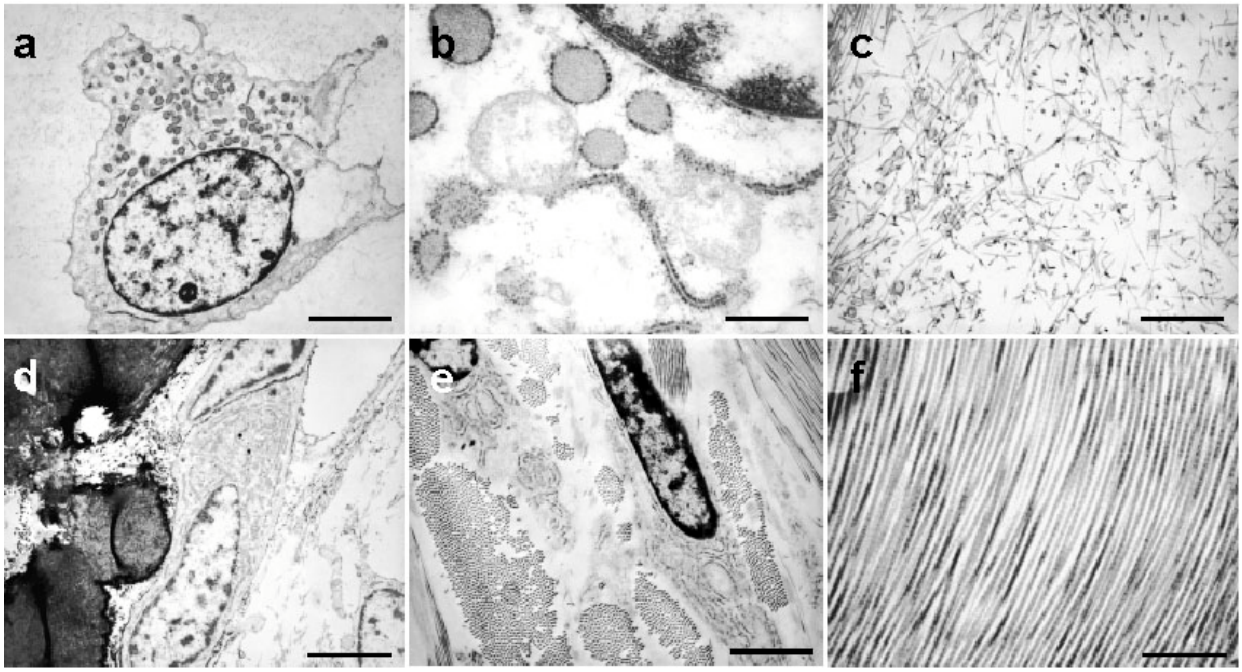

Figure 4. Ultrastructural analysis of fetal growth plate chondrocytes in achondrogenesis type II: visible enlarged cells (a) with distended rER (a; b: detail). The extracellular cartilage matrix shows a very reduced concentration of collagens and interfibrillar ground substance (c). In contrast, osteoblasts (d), fibroblasts (e) and the extracellular matrix of bone (d) and fibrous tissue (e; f: tendon) were normal. (magnification bars: 1 μm).

normal cases (20). Type X collagen was not detectable within the enlarged chondrocytes of the growth plate cartilage (figure 3k) as in the normal cases. However, it was multifocally strongly present around chondrocytes within the bone trabeculae (figure 3 l, m).

4.7. Immunolocalization of interstitial collagen types I, III, and VI

In cartilage staining was observed for collagen types I (figure 3h) and III (figure 3i) nearly throughout all zones. Both were not restricted to the perivascular matrix within the vascular channels. Type VI collagen showed a more inhomogeneous staining (figure 3j) and was only focally concentrated in the perichondrocytic matrix compartment.

In the other connective tissues all three antibodies showed a staining as expected from the physiological situation. Thus, the bone trabeculae, both of the affected and normal cases, were strongly positive for type I collagen whereas collagen types III and VI were largely restricted to the endosteum and to the periosteocytic area. The latter was particularly strong for type VI collagen staining.

4.8. Ultrastructural analysis

In the affected fetuses transmission electron microscopical analysis revealed a very much dilated rER in many fetal chondrocytes in particular of the “hypertrophic” zone, which showed considerable enlarged cells (figure 4a, b). The extracellular matrix was very poor in collagen fibrils and also reduced in proteoglycan content though the latter was less reliable detectable with the technique used (figure 4c). The other types of connective tissue cells as e.g. osteoblasts (figure 4d) or fibroblasts (figure 4e) appeared to be normal and did not show a distended rER. Also the collagenous matrix was unremarkable and showed a regular concentration and arrangement of the collagen fibrils (figure 4f).

5. DISCUSSION

Here, we present the analysis of matrix biochemistry and cell differentiation of achondrogenesis type II (Langer-Saldino) characterized histologically by a largely deranged growth plate cartilage, plump bone trabeculae, numerous vascular channels with perivascular fibrosis and enlarged chondrocytes resembling hypertrophic cells next to the zone of bone formation (10;21). The extracellular matrix appeared to be reduced in volume and consistency. The latter was documented by a very much reduced concentration of collagens and to a lesser extent of proteoglycans (6;11). With morphology-based methods we could clearly show that the reduction in overall proteoglycan content is not restricted to the large vascular channels as suggested previously (6), but a phenomenon directly related to the hyaline cartilage itself. It remains to be elucidated whether the reduction in proteoglycan content is due to a decreased neo-synthesis by the chondrocytes. More likely, it is due to reduced fixation to a normal collagen network and subsequent loss of newly synthesized aggrecan monomers. In this respect, collagen type II fibrils are presumably different from other interstitial collagens which are physiologically not linked to any proteoglycaneous interfibrillar matrix.

Besides the deficiency of collagen of the cartilage

matrix, ultrastructural analysis of our case also showed the distended rER typical of this condition (21-23), most likely related to improper processing and retention of genetically altered type II collagen. Similar phenomena are observed in genetic alterations of Col1 (alpha 1 and alpha 2) chains in osteogenesis imperfecta (24, 25)

Overall, our immunolocalization studies are in line with previous biochemical results, but allowed us to exactly localize the proteins of interest: thus, we could confirm the presence not only of type II collagen (6;10), but also of collagen types I (10;11), III, and VI within the cartilage matrix. Our results do not support the assumption that the type I collagen found biochemically (6;9;10) derives solely from vascular channels, the matrix of which is in fact composed of collagen types I, III, and VI similar to vascular channels of physiological growth plates. Thus, our case is clearly different from a previous case without collagen types I and III in the cartilage (12). However, in this case there was largely normal collagen types II, IX and XI within the cartilage matrix, which was different to other cases (11;26), which showed biochemically no (intact) collagen type II (6;11;27). Obviously, a spectrum of alterations exist leading to a rather similar radiological and histological phenotype. Interestingly, part of the monoclonal antibodies used in this study failed to show significant signals for collagen type II indicating that either the detected epitopes are not present due to the genetic defect or at least the confirmation in the binding region of the antibody is significantly altered.

Besides matrix composition, we were also interested whether the disturbance on the histological level is reflected in disturbed differentiation pattern of the chondrocytic cells. In fact, a regulatory disturbance of chondrocytic differentiation was suspected to be one possible reason for the biochemical alterations of the cartilage matrix, namely a lack of a chondrocytic phenotype of the histologically chondrocytic cells (11). The presence of type II collagen as well as proteoglycans (6) - shown here to be cartilage-specific aggrecan - throughout the cartilage matrix indicates chondrocytic differentiation of the cells, which is also supported by the expression of S-100 protein (28). However, collagen type IIA, a splice variant physiologically expressed specifically by chondroprogenitor cells was found throughout the cartilage matrix and not restricted to the epi- and perichondral zones suggesting that COL2A might be expressed in most chondrocytes in achondrogenesis indicating a delay in chondrocyte maturation. In fact, the lack of expression of type X collagen in the areas of enlarged chondrocytes ("hypertrophic" chondrocytes (10)) further supports the concept of delayed chondrocytic differentiation during the disease process. The presence of type X collagen within the bone trabecules confirmed, however, the principle capability of the cells to express this biological marker of hypertrophy (29). Cellular enlargement, the characteristic sign for physiological chondrocyte hypertrophy in conventional chondrocytes is in the context of achondrogenesis type II biologically misleading as it is not related to a cellular differentiation program, but at least in part due to a cytoplasmic enlargement due to a dilatation of rER by wrongly processed collagen type II molecules as discussed above.

Interestingly, there exist animal models closely resembling the features found in achondrogenesis type II (for review see (30)): Col2A-null mice are in this respect, however, different as they fail to show not only type II collagen, but also show clear hypertrophic differentiation of epiphyseal cells with the expression of type X collagen (31): whether this indicates that a deranged type II collagen is more detrimentous than a missing type II collagen gene requires further experiments. Bovine achondrogenesis (32) resembles very much the human condition as both show extensive vascular channels and the presence of type I collagen in biochemical analysis. Whether type I collagen in bovine achondrogenesis comes only from the cartilage channels (32) or also the cartilage itself was never investigated. Also it remained unclear, whether type X collagen is expressed by the enlarged cartilage cells and in the light of our study it appears very questionable whether cellular enlargement in this condition can be taken as a safe indicator for hypertrophic differentiation of chondrocytes (32). The Dmm (disproportionate micromelia)-mice, which lack three nucleotides in the C-propeptide of collagen type II of one allele show also much reduced immunostaining for collagen type II as well as a dilated rER (33). Of interest, the stable transfection of human fetal chondrocytes with a type II procollagen minigene also induces an irregular cell shape, dense intracellular granules, and less collagen in the newly formed extracellular matrix (34).

Unclear is the heritage of achondrogenesis type II. Whereas it seems to be clear that most (if not all) cases derive form mutations in the type II collagen gene (for review see (35)) and therefore this condition represents an autosomal disorder, most published cases indicate a dominant trait (9;36), whereas others as ours indicate a recessive one. On the molecular level many different alterations were described implicating different biochemical hazards. Thus, (heterozygous) single base mutations (Gly310->Asp (9); Gly691->Arg (23)) lead to an overmodification of type II collagen with reduced thermal stability of the collagen type II fibril and a delay in collagen type II secretion at least *in vitro*. Other mutations in the COL2A1 gene lead to a wide range of phenotypes from lethal forms (achondrogenesis type II and hypochondrogenesis) to severely affected patients (Kniest dysplasia (37), spondyloepiphyseal dysplasias) and mildly affected people (Stickler syndrome (38)).

Altogether, for achondrogenesis type II a genetic defect in the type II collagen gene, either heterozygous in a dominant fashion or homozygous in a recessive trait, leads to a default in proper processing of type II collagen and often to an overmodification of the collagen type II fibril (6;9). All this implicates an impaired processing of newly synthesized collagen type II fibrils in the rER leading to an accumulation and retention of altered collagen type II molecules with dilation of the rER (for review see (30)). Subsequently, the extracellular matrix is lacking type II collagen in amount and functionality which itself presumably inhibits proper proteoglycan fixation and

deranges architecture and cellular differentiation pattern. Altogether, the alterations are so severe that they are not compatible with proper growth of the skeleton and survival of the individual.

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