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Hyaline Cartilage Changes in Diastrophic Dwarfism

Max Scheck[1], Jan Parker[1], and Donna Daentl[2]

[1] University of California, San Francisco, Department of Orthopaedic Surgery, Orthopaedic Surgical Laboratory, 641 HSE, San Francisco, California 94143, USA

[2] University of California, San Francisco, Depts, of Growth and Development, Pediatrics, and The Center for Craniofacial Anomalies, San Francisco, California 94143, USA

Summary. Hyaline cartilage of the talus of a diastrophic dwarf was studied by light and transmission electron microscopy before and after proteoglycan extraction or digestion, glycogen digestion, and enzyme marking.

The nuclei of the chondrocytes were as a rule large and round and the cytoplasm contained large vacuoles. Best's carmine stained the cytoplasm of most cells red; after diastase digestion the cytoplasm remained unstained. This suggested that the cells contained glycogen. This observation was complimented by the ultrastructural demonstration of large amounts of glycogen. Cell scars were frequent. The shape and state of activity of the cells as well as the development of intracytoplasmic organelles showed great variability. The matrix showed many areas of degeneration and a general dearth of sulphated acid mucopolysaccharides. A lacey pattern of unmasked collagen fibers was frequently seen. Collagen fibers showed a great variability in diameter and often appeared frayed when examined by electron microscopy.

These observations suggest an enzymatic deficiency in chondrocyte mucopolysaccharide and glucose metabolism. The techniques we used when added to biochemical studies should prove useful in the investigation of human dwarfism.

Key words: Hyaline cartilage – Proteoglycans – Glycogen – Matrix degeneration – Electron microscopy.

Introduction

Diastrophic dwarfism is an autosomal recessive disorder. Short limbs, hip contractures, clubfoot, clubhand, scoliosis, swelling and redness of the ear pinnae are the more common clinical manifestations. The skull is usually spared. An-

For offprints contact Dr. M. Scheck

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other distinguishing feature is hyperextension of the thumb or "hitchhiker's thumb." The histologic changes of iliac apophysis or rib cartilage have been described by Rimoin et al (1974) and ultrastructural epiphyseal plate changes have been recorded by Chollak et al. (1976).

This report concerns the morphological and histochemical changes of hyaline cartilage of the talus of a diastrophic dwarf. The tissue was obtained at the time of operative correction of a severe clubfoot deformity. The cartilage was studied by light microscopy and electron microscopy before and after proteoglycan extraction or digestion, glycogen digestion, and enzyme marking.

Case Report

N.B. was born to normal, non-consanguinous parents following an uncomplicated pregnancy. She had short limbs, clubfeet and hip flexion contractures. At the age of three months, redness and swelling of one ear pinna was noted. This was followed by calcification. An operation for clubfoot correction was done at the age of nine months and a cartilage slice was submitted for histological examination. Because correction was not successful, a talectomy was performed in August of 1977 when the child was 28 months old. Ample hyaline cartilage became available, enabling us to carry out the previously described examinations.

Materials and Methods

Talar cartilage was fixed at the time of surgery in 10% formalin, absolute alcohol, or Karnovsky's mixture.

Several fresh tissue slices were immediately frozen in liquid nitrogen and stored at $-70°$ for about 6 weeks, until the histochemical procedures were started.

Electron Microscopy

Fresh tissue slices were immediately immersed in cold 4% paraformaldehyde-2% glutaraldehyde buffered with 0.2 M sodium cacodylate, pH 7.4, with $CaCl_2$ added (Karnovsky, 1965) and stored at 4° C for one month before postfixation in either 2% aqueous $KMnO_4$ or cacodylate buffered 1% O_sO_4, pH 7.4, for $1^1/_2$ h. After a buffer rinse the osmicated tissue was stained en bloc with 2% $KMnO_4$ for 30 min, 1.5% uranyl acetate in 50% ethyl alcohol for 15 min, or 1% PTA in absolute alcohol for 30 min. Some frozen tissue was handled the same way after 2 h primary fixation. Other of the frozen cartilage was fixed in 2% KM_nO_4 and dehydrated and embedded without further treatment.

All tissues were dehydrated in ethyl alcohol, infiltrated with propylene oxide and plastic and embedded in Spurr's medium (Spurr, 1969). Thin sections were photographed in a Zeiss EM 9A without further staining or after contrasting with uranyl acetate and lead citrate (Reynolds, 1963).

Extraction. Thin slices of frozen cartilage were subjected to 4M guanidinium chloride in 0.1 M sodium acetate-acetic acid, pH 4.0, or to the buffer only for 7 days at room temperature. Solutions were changed daily (Campo and Phillips, 1973). Decreasing salt concentrations (4 M, 2.5 M G-Cl) were applied during the last 12 h of treatment (Orkin et al., 1977). Both extracted and control tissue was then fixed for 2 h in Karnovsky's mixture or Karnovsky's with 0.2% Ruthenium red (RR) and postfixed in O_sO_4 or O_sO_4 + RR.

Hyaluronidase Digestion. Fresh frozen tissue was incubated with agitation for 1 h at 37° C in 3000–4000 Iu/ml bovine testicular hyaluronidase (Sigma Chemical Co.) in 0.154 NaCl, pH 6.0. Controls were incubated in buffer only. After digestion the cartilage was fixed in either Karnovsky's fixative or Karnovsky's + RR (Thyberg et al., 1973).

Some frozen tissue was *fixed* for 2 h with or without RR prior to 18 h digestion with agitation at 37° C in 0.1 M cacodylate buffered enzyme, pH 5.4. Controls were incubated in buffer alone (Dearden and Bonucci, 1975).

All tissues were postfixed in O_sO_4 or O_sO_4 + RR.

Amylase Digestion. Slices of frozen tissue were fixed for $1^1/_2$ h in either Karnovsky's or 2% KM_nO_4 prior to 2 h incubation with agitation at 37° C in 0.1 M cacodylate buffer, pH 7.3, with or without 0.5% bacterial crude alpha amylase (Sigma Chemical Co.) (Ermak, 1977). All tissues were postfixed in O_sO_4.

Thin sections of routinely prepared and embedded tissue were digested with 0.5% alpha amylase in 0.2 M acetate buffer, pH 5.6, for 12 h (Ohyumi and Takano, 1977).

Acid Phosphatase Reaction. Frozen tissue was fixed for 30 min or 2 h in Karnovsky's mixture, sliced as thin as possible with a razor blade, washed in several changes of 0.1 M cacodylate buffer, pH 7.2, and then in 0.05 M acetate buffer pH 5.0. The tissue was incubated at room temperature for 15 min or 1 h in the sodium beta glycerophosphate medium described by Zimmerman et al. (1976). The medium was made up and filtered immediately prior to use and changed once during incubation. Controls were incubated without the glycerophosphate. Tissues were washed briefly in 0.1 M acetate buffer, pH 5.0, and then in distilled water prior to 30 min postfixation in O_sO_4, dehydration, and embedding (Christie and Stoward, 1977).

Light Microscopy

Frozen tissue which had been fixed in Karnovsky's before or after guanidinium extraction of hyaluronidase or amylase digestion, as previously described, and formalin and alcohol fixed material were embedded in paraffin. Frozen tissue was also fixed in cold acetone and embedded. Six micra sections were cut and stained with 1) H&E 2) Safranin O 3) Alcian blue 4) PAS 5) PAS after diastase digestion.

Frozen tissue was also fixed in cold acetone, embedded, sectioned and incubated in Gomori's lead nitrate for acid phosphotase (Gomori, 1952).

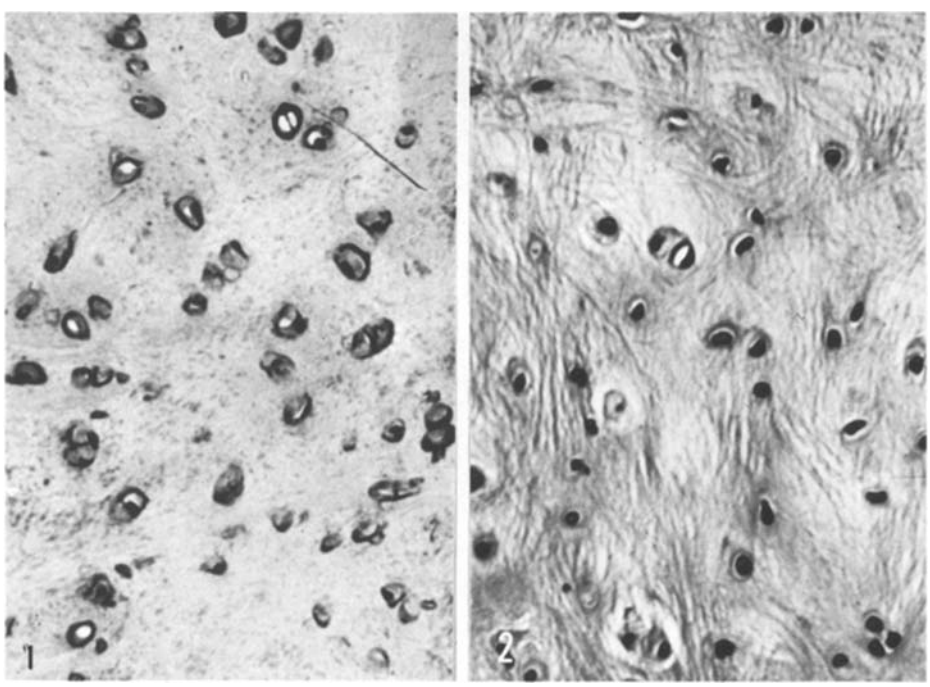

Fig. 1. Rings of stained substance surround the chondrocytes, which are either single or in pairs. The interterritorial matrix is pale and practically unstained (Alcian blue, ×250)

Fig. 2. In some areas the matrix fibers form a lacey pattern (PAS, ×250)

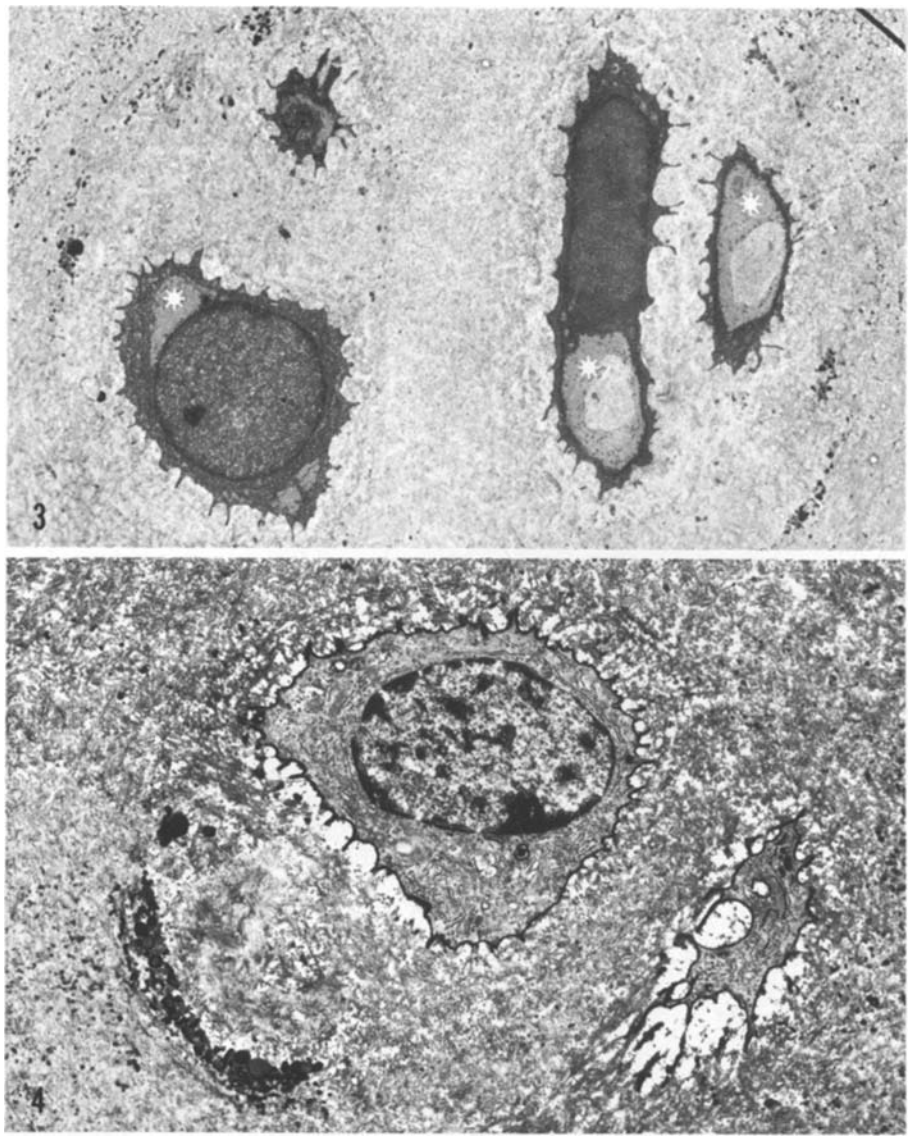

Fig. 3. Differently shaped chondrocytes. The cells contain much glycogen (*). Postfixation with osmic acid and potassium permanganate, ×4750

Fig. 4. Cartilage cell with well developed organelles. Note the accumulation of dense material at the plasma membrane. A pyknotic remnant of a cell is seen in the left lower corner and a hypertrophic cell in the right lower corner. Postfixation in osmic acid and stained en bloc with uranylacetate, ×6450

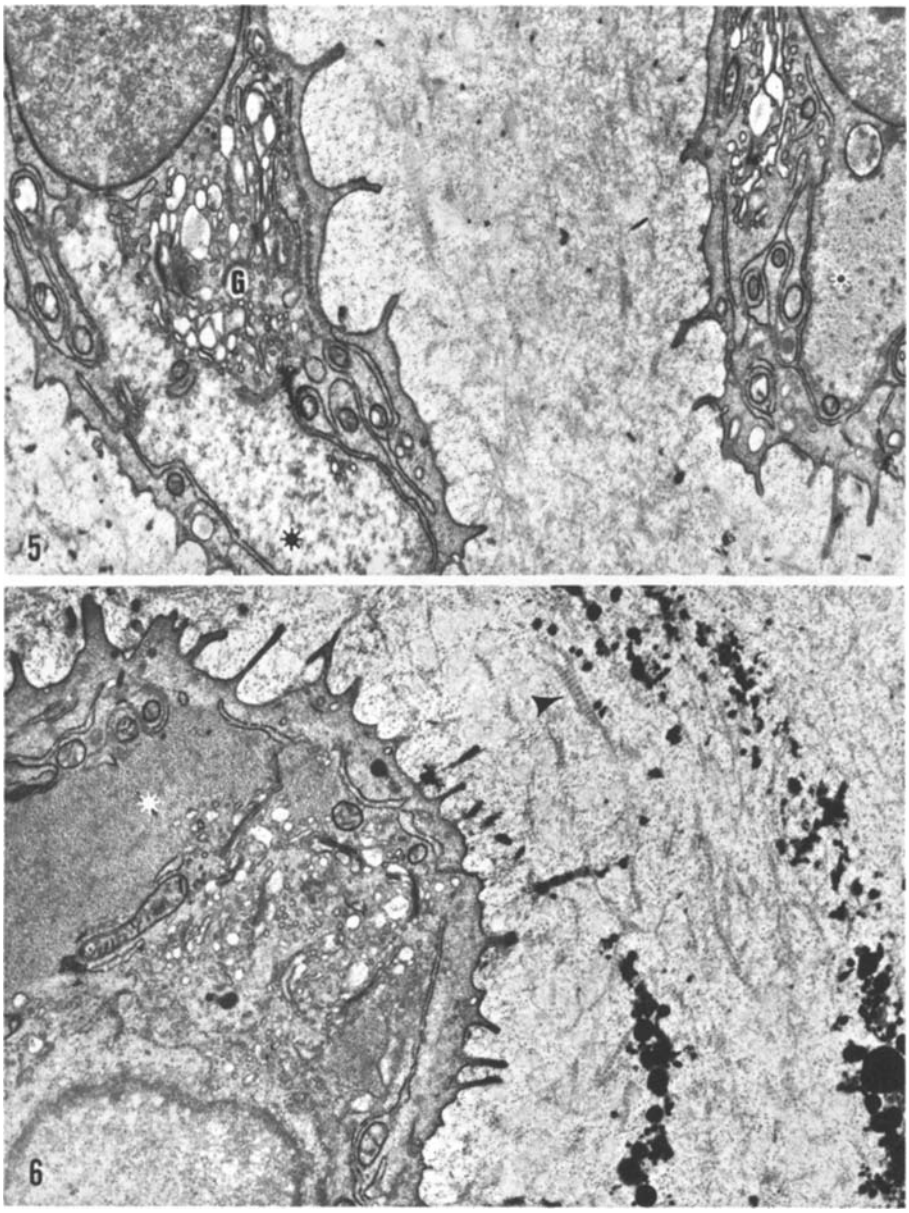

Fig. 5. Chondrocytes with glycogen (*) in different forms and prominent Golgi (*G*) zones. The glycogen of the cell on the left is in floccular form whereas a granular form is seen in the cell on the right

Fig. 6. Glycogen in granular form (*). Many electron dense bodies are seen in the matrix and the collagen fibers are unmasked and show good banding (*arrowhead*)

Figs. 5 and 6. Osmicated tissue postfixed in potassium permanganate, × 13,500.

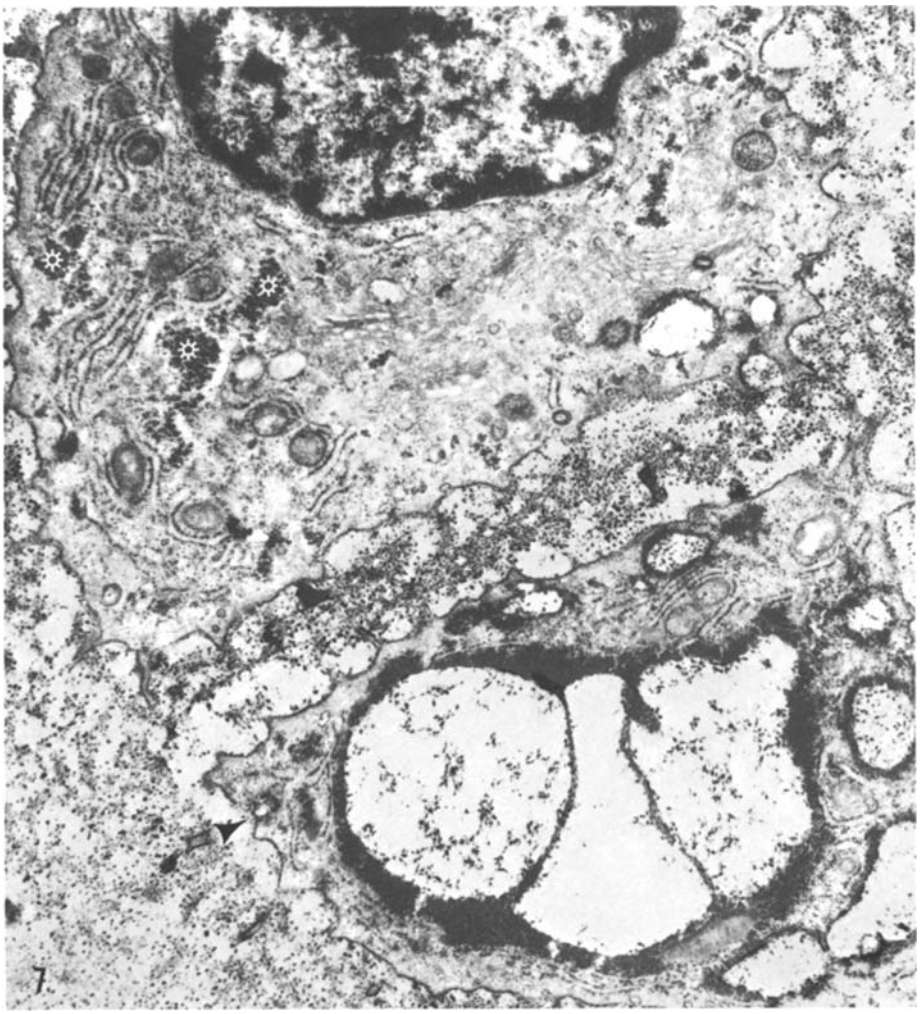

Fig. 7. Glycogen rosettes in several areas of the upper chondrocyte (*). Well developed RER, round mitochondria, a centrosome and pale staining Golgi are seen. Pinocytotic vesicles are rare. The lower cell shows a large glycogen area with electronlucent interior and accumulation of dark staining granules at the periphery. Smaller glycogen areas are seen close to the cell membrane and exocytosis is apparently taking place (*arrowhead*). There is granular material between the cells and also in the surrounding matrix. Postfixation with osmic acid and en bloc staining with PTA, ×15,000

Results

Light Microscopy

Nuclear staining of the chondrocytes was preserved but occasional cell ghosts were seen. The nuclei were large and round and the cytoplasm often contained large vacuoles. The cells were mostly single, but occasionally doublets and

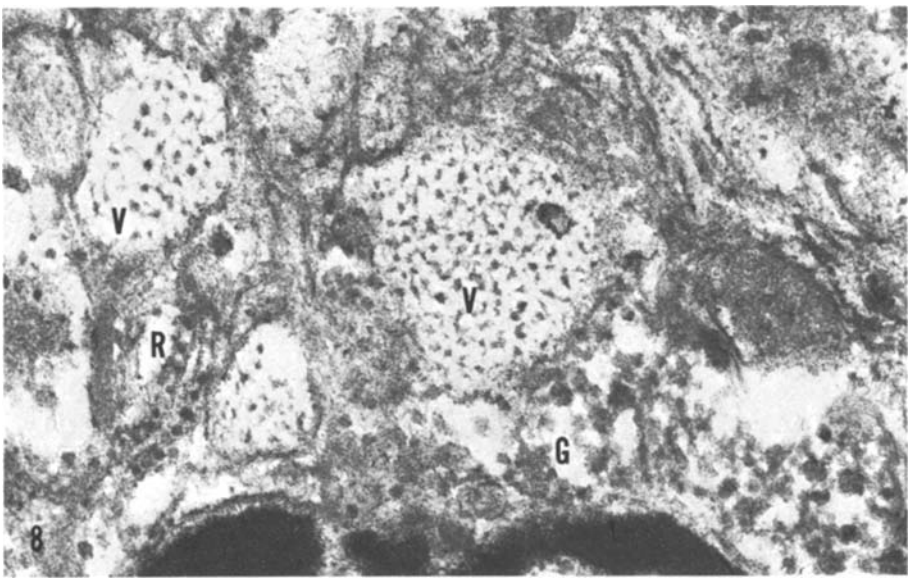

Fig. 8. Large vacuoles containing stellate or polygonal particles (*V*). Adjacent to one of the vacuoles is a large glycogen area (*G*). A portion of endoplasmic reticulum lined with polyribosomes (*R*) is seen on the left. Note the difference in particle size and electron density. Tissue incubated in buffer as a control for hyaluronidase digestion and stained with Ruthenium red, × 56,700

clusters were seen. When stained with Best's carmine, the cytoplasm of many cells stained red. When stained after digestion with diastase, the cytoplasm was pale, suggesting that the red staining substance was glycogen.

The matrix contained cell scars. Using staining methods specific for sulfated proteoglycans (Alcian blue, Safranin O) rings of stained substance were seen surrounding the chondrocytes but there was practically no staining of the interterritorial matrix (Fig. 1). At times a lacey pattern of matrix fibers, as described by Rimoin (1974) was seen (Fig. 2). Dissociative extraction of proteoglycans by guanidinium hydrochloride had little effect on the intensity of matrix stain regardless which stain was used; however, the extraction affected the width and the intensity of the stain immediately surrounding the chondrocytes. The non-extracted sections showed a much heavier ring of stained material. Similar qualitative observations with regard to differences in staining intensity were made when hyaluronidase was used for digestion of chondroitin sulfate. The greatest difference in staining intensity was found when a-amylase had been used for digestion of glycogen, glycoprotein and proteoglycan (Quintarelli, 1969). The intensity of stain with the PAS method was markedly reduced after a-amylase digestion.

Electron Microscopy

Different methods of postfixation and staining enabled us to study the cells and matrix in detail. Potassium permanganate proved to be an excellent fixative

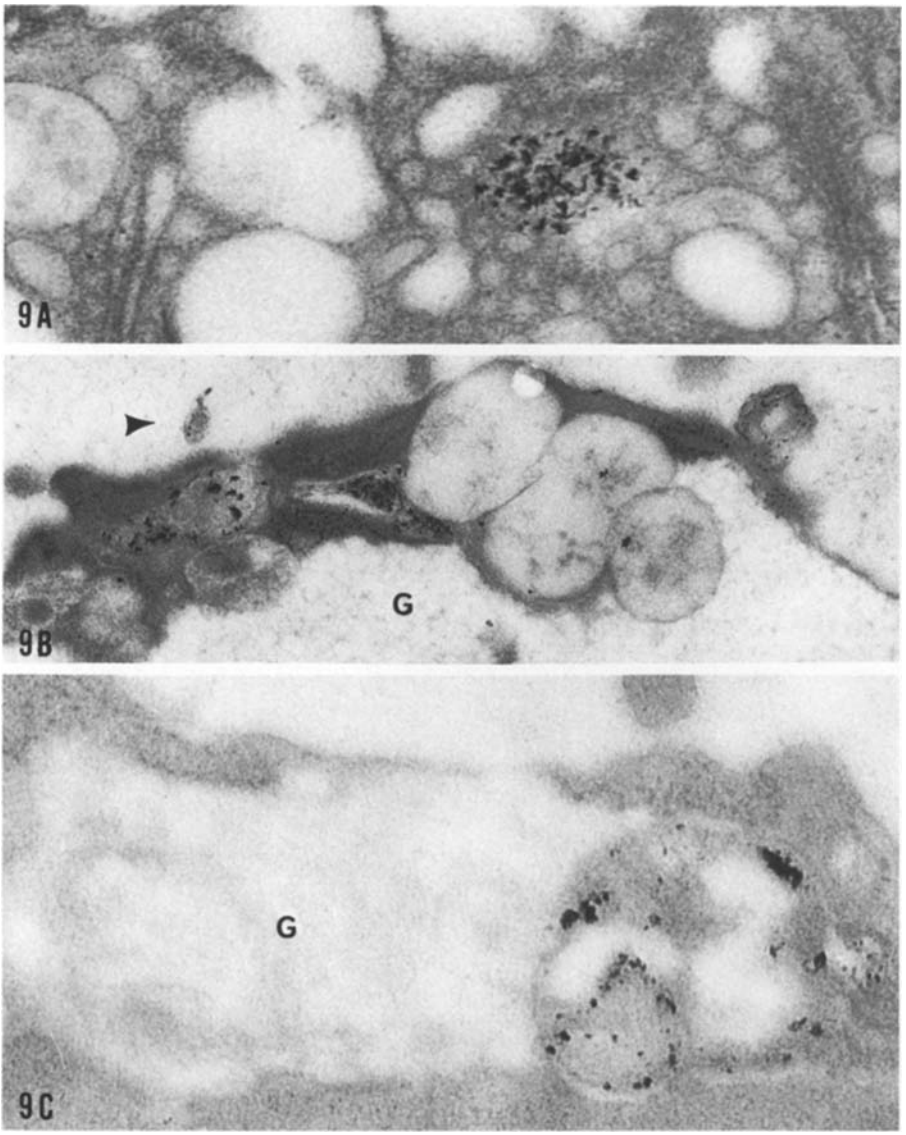

Fig. 9A–C. Tissue incubated in sodium beta glycerophosphate, ×67,500. **A** Lysosome containing reaction product within the Golgi area. **B** Vacuoles containing reaction products at the periphery of the cell surrounding a glycogen lake (*G*). A small body containing reaction product is seen in the matrix close to the cell (*arrowhead*). **C** Cytolysosome adjacent to amorphous glycogen area (*G*) at the periphery of a cell

for glycogen, and enhanced membrane definition. PTA, at the acid pH we employed, stained proteoglycan and glycogen well. A great variability in the shape and state of activity of the chondrocytes was found. Some cells had a large round nucleus with regular chromatin distribution. Other cells had an oblong shape and irregular nuclei (Fig. 3). About half of the cells had promi-

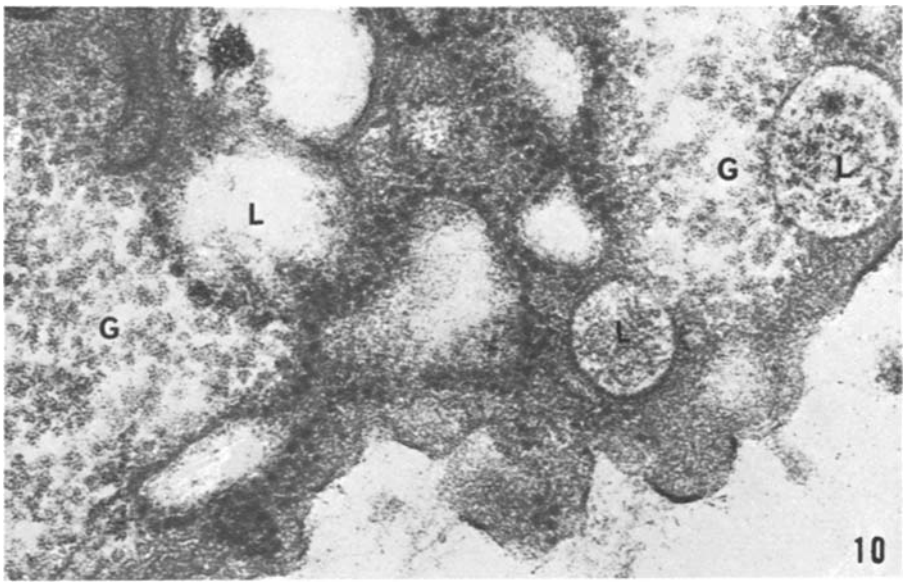

Fig. 10. Cytolysosomes (*L*) surrounding glycogen lakes (*G*). Postfixation with osmic acid and stained en bloc with uranyl acetate, ×67,500

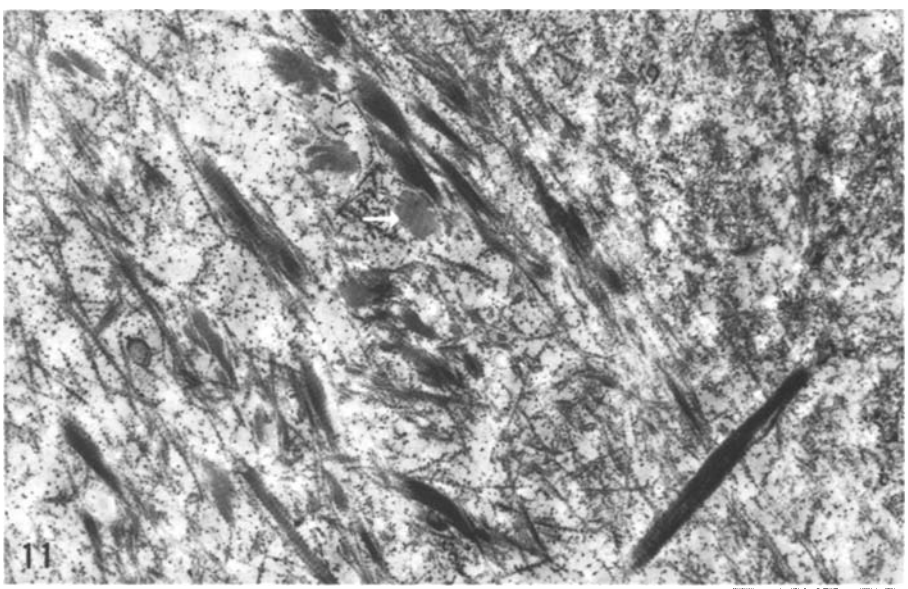

Fig. 11. Great variability of matrix collagen fiber size. Tactoids (*arrow*), granules and fine filaments are also seen. Postfixed with osmic acid and stained en bloc with uranyl acetate, ×12,000

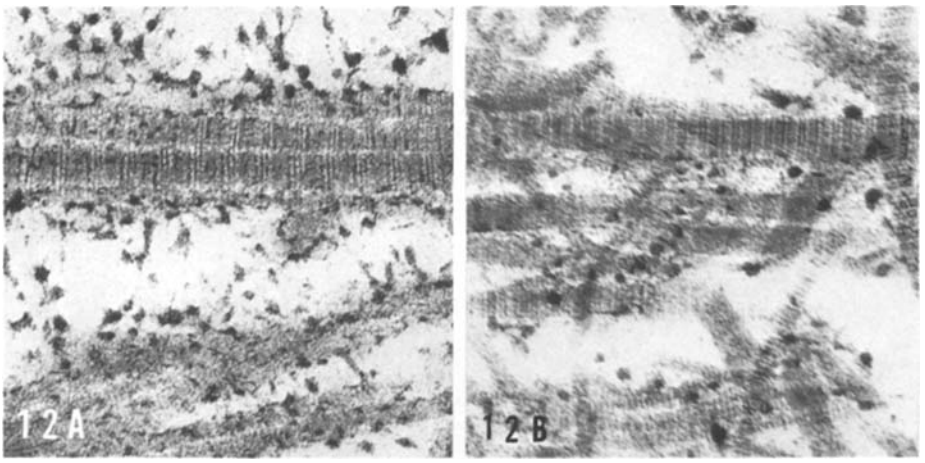

Fig. 12. A Polygonal densities as well as smaller granules with attached fibrils. Osmic acid-Ruthenium red postfixation, ×67,500. **B** Frozen cartilage extracted with guanidinium chloride. Only partial extraction was accomplished. Some proteoglycan is still present but the collagen fibers are unmasked and show distinct banding (after extraction with guanidinium chloride and postfixation with osmic acid-Ruthenium red, ×67,500

nent nucleoli. The development of intracytoplasmic organelles varied with some cells showing much rough endoplasmic reticulum and little Golgi whereas the proportion was reversed in others. Free ribosomes or lipid were not seen. Intracytoplasmic filaments were abundant. The mitochondria were mostly of normal size and electron density but in some cells they were large and pale. The chondrocytes had no lacunae. When stained with uranyl acetate dense material was seen accumulated at the plasma membrane. Many cells were hypertrophic or pyknotic and debris was seen in the matrix (Fig. 4). Postfixation in osmic acid and en bloc staining with potassium permanganate or PTA demonstrated an unusual amount of glycogen in the majority of chondrocytes. The glycogen was either in granular, rosette or floccular form (Figs. 5 and 6). In other cells large areas with pale centers and accumulation of glycogen granules at the periphery were seen (Fig. 7). Membrane bound vacuoles were of two types which could be distinguished because of our use of different techniques of preparation. Ruthenium red was used to demonstrate proteoglycan in the form of stellate or polygonal particles in some of the vacuoles. We therefore believe that they are large Golgi vacuoles (Fig. 8). The other type of vacuole was identified as being of lysosomal origin by acid phosphatase reaction (Figs. 9A to C). These vacuoles were seen either at the periphery of the cell adjoining glycogen areas (Fig. 9B and C) or occasionally in the midst of the Golgi zone (Fig. 9A). The proximity of cytolysosomes to areas with stained glycogen is also shown in Figure 10.

Matrix. Many areas of degeneration were seen. The collagen fibers showed great variability in diameter; fraying of fibers and tactoids were observed (Rowsey et al., 1976). The spaces between the fibers were large and showed

much granularity (Fig. 11). When stained with Ruthenium red, polygonal densities attached to certain band sites of the collagen fibers, as well as smaller granules with attached fibrils, were seen (Fig. 12A). Exposure of slices of frozen cartilage to guanidinium chloride led only to partial extraction of proteoglycans (Fig. 12B). Hyaluronidase and amylase digestion studies were inconclusive.

Discussion

Histological, histochemical and ultrastructural examination of the hyaline cartilage indicate a deficiency of proteoglycans of the matrix and degenerative changes. The tissue had been obtained from the talus of a clubfoot which had been subjected to attempts of correction in the past. Treatment included immobilization of the foot in a forced position and this could have resulted in cartilage damage (Salter and Field, 1960). However, we do not believe that the changes we observed are iatrogenic. Quantitatively, the chondrocytes showed no unusual loss of nuclear staining and the histologic changes of the matrix are, in essence, similar to the changes described by Rimoin et al. (1974) in untreated rib and iliac crest cartilage biopsies of diastrophic dwarfs.

In the majority of chondrocytes both light and ultrastructural microscopy show a large amount of glycogen. Judging from the development of cytoplasmic organelles, the majority of chondrocytes looked healthy and not hypertrophic or dying. Glycogen is normally found in chondrocytes of epiphyseal cartilage, but we believe that in this instance, the amount is unusually large. It has been suggested (Godlewski, 1971) that the accumulation of unusual amounts of glycogen is indicative of low oxidative activity of the cell. Whereas the available glucose is immediately utilized in an active oxidative system, glucose accumulates in cells with low oxidative activity and is converted to glycogen. Whether in this instance the accumulation of glycogen in the chondrocytes is due to a defect of the oxidative system of the cell is open to speculation. In this connection, biochemical studies of achondroplastic rabbits and experiments with induced micromelia of chicks are noteworthy. Shepard (1971) found a metabolic defect in glucose utilization in organ culture studies of achondroplastic rabbit cartilage. Incorporation of 14 C from glucose by the dwarf cartilage explants was increased significantly. The dwarf nuclear area was greater than that of the control. The author thought that the increase in size of the nucleus and the increased 14 C incorporation may be indicative of an increase in the manufacture of messenger ribonucleic acid, a mechanism whereby the cell unsuccessfully attempted to correct an enzyme deficiency. Bargman et al. (1972) found that a defect involving aerobic energy formation is present in liver mitochondria from achondroplastic rabbits with the absence of ATP formation at the cytochrome oxidase region (site 3) of the terminal transport chain. The authors suggested that in tissues in which oxygenation is normally low, such as cartilage, the defect of oxidative phosphorylization may cause limitation of growth and development by reduction of oxidative energy formation. Landauer (1969) concluded that disturbance of carbohydrate metabolism is one of the mechanisms by which many teratogens induce limb malformations.

Rimoin et al. (1974) have suggested that the basic defect in diastrophic dwarfism may be an enzymatic deficiency in chondrocyte mucopolysaccharide metabolism. Our morphological observations not only show a quantitative reduction of proteoglycans in the matrix but the sulfated acid mucopolysaccharide-protein complex may also be qualitatively different. Although the tissue slices were subjected to dissociative extraction with guanidinium chloride for 7 days, extraction of proteoglycan was only partial and much less than the anticipated rate of 80–90% (Campo, 1973). This may be due to an alteration in the structure of the proteoglycans. The accumulation of glycogen in chondrocytes which we found may be indicative of a defect in glucose metabolism. To our knowledge, biochemical studies of diastrophic dwarf cartilage have not been carried out.

Biochemical studies of cartilage of diastrophic and other forms of human dwarfism combined with histochemical methods and the examination of the ultrastructure may help to identify factors responsible for the inability of cartilage to grow and develop in a normal manner.

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