STUDIES ON ULTRASTRUCTURAL IDENTIFICATION AND DISTRIBUTION OF PROTEIN-POLYSACCHARIDE IN CARTILAGE MATRIX

VICTOR J. MATUKAS, BERNARD J. PANNER, and J. LOWELL ORBISON

From the Department of Dentistry and Dental Research and the Department of Pathology, University of Rochester, Rochester, New York. Dr. Matukas' present address is the Department of Pathology, University of Pennsylvania, School of Dental Medicine, Philadelphia

ABSTRACT

Previous reports on the ultrastructure of cartilage matrix have described fibers, amorphous ground substance and, in some instances, dense matrix granules. The fibers are presumably collagen, but the nature of the granules is unknown. The primary purpose of this study has been to investigate the ultrastructure of cartilage matrix in chick embryos with particular emphasis on the distribution and composition of these granules. In matrix of the zone of articular cartilage, mature collagen fibers can be seen but granules are not present. In matrix of all other zones of cartilage, fibers are smaller and granules are present. When the matrix of epiphyseal cartilage is compared to that of the zone of hypertrophic cells, fibers are similar but the granules in the latter zone are larger and more numerous. The granules in both zones were digested by hyaluronidase and positive to colloidal iron staining. Chemical analyses of cartilage from these zones indicate the hexosamine and radiosulfate content of the zone of hypertrophic cells to be higher than that of the zone of epiphyseal cartilage. The increased hexosamine was shown by column chromatography to be principally sulfated mucopolysaccharide, thereby indicating a direct correlation between size and number of granules and sulfated mucopolysaccharide content in the two zones. These data and the results of the electron microscopic histochemical studies are consistent with the concept that the granules in cartilage matrix contain acidic mucopolysaccharide.

INTRODUCTION

All previously reported ultrastructural studies of cartilage have described the matrix as containing a network of fine fibers which are presumably collagen (20, 13, 21, 40, 44, 50, 15). Occasional banding has been reported in these fibers but there was no evidence of 640 A periodicity. Mention of another component of cartilage matrix represented by "round structures varying from 100 to 500 A in diameter" was made by Robinson and Cameron (40). They postulated that these structures may represent tubules and suggested the possibility of a canalicular system between cartilage cells (2). This granular component was also described by Takuma (48) and by Godman and Porter (13). The latter authors pointed out that these matrix granules are distinguishable from the cross-section of fibers by their greater electron density and larger size and suggested that they may represent mineral deposits. Revel and Hay (38) also reported dense material along the meshwork
of fibers in cartilage matrix and postulated that this material and the amorphous material between fibers may be mucoprotein.

In the present study we have investigated the ultrastructure of cartilage matrix in developing chick tibia, using various fixatives and electron microscopic histochemical techniques. These ultrastructural studies were correlated with microchemical analysis of the different cartilaginous zones. Special effort has been made to elucidate the nature and structure of matrix granules and to determine their relationship to fibers.

**MATERIALS AND METHODS**

**Morphology**

Chick embryos (Spafas) were incubated at 37°C for 14-16 days. In all cases the distal end of the tibia was used for sampling. Tibial cartilage was divided into zones according to Fell (10) (Fig. 1), and sample blocks were taken from each zone.

For ultrastructural studies 1 mm³ blocks were immediately placed in one of the following fixatives: (a) Veronal-buffered 1% osmium tetroxide for 1.5 hr (31, 4); (b) phosphate-buffered 4% formalin for 1.5 hr (36); (c) phosphate-buffered 4% glutaraldehyde for 1.5 hr (41); (d) Lillie's lead nitrate for 20 hr (17).

The above fixatives were also used in combination with cetylpyridinium chloride (CPC), an anionic detergent commonly used for precipitating acid mucopolysaccharides in chemical investigations (53). Usually fixation was carried out at 4°C but, when CPC was added, the fixation was done at room temperature to avoid precipitation of the CPC. All tissues were postfixed in Veronal-buffered osmium tetroxide for 1 hr, with the exception of the lead nitrate-fixed tissue which was immediately placed in 70% ethanol. Blocks were dehydrated in a graded series of alcohols and embedded in Epon 812 (18). Sections were cut at approximately 800 Å on an MT-1 Porter-Blum microtome, picked up on carbon-coated 200-mesh grids, and stained with either lead citrate (39), uranyl acetate (52), or phosphotungstic acid (52) for examination in an Hitachi HU 11-A electron microscope. In some cases double staining with uranyl acetate and lead nitrate was used. 1 M sections were also stained with 1% toluidine blue in 1% borax for light microscopy.

**Electron Microscopic Histochemistry**

**Enzyme Digestion**: Blocks of tissue were obtained as previously described, fixed in phosphate-buffered 4% formalin for 3 hr in the cold, and washed in plain phosphate buffer. Experimental blocks were placed in either phosphate-buffered hyaluronidase (150 µ/㏄) or trypsin (3 mg/ml). Control blocks were placed in phosphate buffer or in a comparable enzyme solution inactivated by boiling for 1 hr. All blocks were then incubated for 12 hr at 37°C. After removal from the incubator, blocks were postfixed in 1% osmium tetroxide (28) and handled as previously described.

**Colloidal Iron**: Attempts at utilizing a previously described modification of colloidal iron staining for electron microscopy (6) were unsuccessful. Poor penetration of the iron particles consistently resulted when small blocks of tissue or 50 µ frozen sections were incubated in Mowry's colloidal iron solution (29). In every case a narrow rim of staining took place around the periphery of the blocks, with no evidence of iron uptake in central areas.

In order to overcome this difficulty, thin sections mounted on carbon-coated grids were inverted on a drop of Mowry's modification of colloidal iron for 1 hr. These sections were then rinsed by dipping in three changes of 12% acetic acid and two changes of distilled water. The Prussian blue reaction was not used since large crystals resulted and abolished the precise localization of the reaction. Colloidal iron was visualized in sections as approximately 30-50 Å diameter electron-opaque particles. Sections were examined directly after colloidal iron staining or after additional staining with uranyl acetate.

**Chemistry**

Fourteen-day-old chick embryos were injected with 50 µ of S³⁵-labeled sodium sulfate and sacrificed 48 hr later. Samples were obtained from the epiphysis and from the zone of hypertrophic cells under a dissecting microscope. They were subjected to several changes of acetone followed by vacuum
desiccation and weighed on a Cahn Electrobalance to determine the dry weight.

Dry tissue weighing between 800 and 1800 μg was digested in conical bottomed Pyrex centrifuge tubes with 1.0 ml of a digest mixture containing 1% papain, cysteine-HCl, and EDTA according to Antonopoulos et al. (1). After the tissue was digested for 3 hr, the tubes were centrifuged for 90 min at 3800 rpm at room temperature in an ordinary centrifuge with a swing-out head. After centrifugation the supernate was carefully removed and transferred to another tube. At this point a small amount of residue remained but was found to contain no detectable hexosamine. Aliquots of supernatant fluid, representing approximately 500 μg of dried tissue, were then fractionated on micro columns (1), sequentially using as eluting solvents 1% CPC, 0.3 M NaCl, 0.3 M MgCl₂, 0.4 M MgCl₂, 0.5 M MgCl₂, 0.65 M MgCl₂, and 6 N HCl. The eluted fractions were analyzed for hexosamine (1) and examined for radioactivity. 50 μl of the supernatant fluid were hydrolyzed in 1.0 ml of 6 N HCl for 8 hr at 100°C in a teflon-lined, screw-topped tube. A 10-μl aliquot of the hydrolysate was dispersed in 100 μl of distilled water on a stainless steel planchet and dried under an infrared heat lamp and counted on a Nuclear Chicago D-47 automatic gas flow counter with a ½mil window and corrected for background. The remainder of the hydrolysate was evaporated to dryness in a vacuum desiccator and hexosamine determined (1).

RESULTS

Electron Microscopy

Ultrastructural characteristics of cells in the various zones of epiphyseal cartilage have been described previously (13) and will be mentioned here only briefly. In articular cartilage, cells resemble fibroblasts. They are spindle shaped, displaying a prominent Golgi apparatus, and contain dilated, well-developed endoplasmic reticulum (Fig. 2). Cells of epiphyseal cartilage are chondroblasts. The cytoplasm is dense and a characteristic lacunar space is evident (Fig. 3). At higher electron microscopic magnifications these granules appear to have a substructure (Fig. 9), and many of these latter structures vary in width from approximately 15 to 40 A. In many areas the matrix changes markedly. The first change is from an ordered array of fibers to a random distribution (Fig. 6), followed by a second area where there is a mixture of mature fibers and smaller fibers measuring approximately 200 A in diameter, (Fig. 7). In this intermediate area a few dense granules are found in the matrix, both associated with smaller fibers and lying free in interfibrillar areas. In epiphyseal cartilage all fibers of the matrix measure about 200 A in diameter (Fig. 8). There is no evidence of 640 A periodicity. When periodicity can be seen it measures about 210 A. The previously mentioned granules are numerous in this area, measuring from 200–400 A in diameter.

There is a change in the character of matrix between the epiphyseal area and the zone of flattened and hypertrophic cells (Fig. 9). In the latter two zones fibers are similar to those seen in the zone of epiphyseal cartilage but granules are larger, measuring from 200 to 700 A in diameter. Many of these granules have small, stellate projections which have approximately the same morphologic and staining characteristics as the fibers (Fig. 9). In some areas granules are concentrated at the interface between lacunae and matrix (Fig. 9). In adjacent 1 μ sections stained with toluidine blue, this interface is seen as a dense metachromatic line outlining the lacunae.

At higher electron microscopic magnifications granules appear to have a substructure (Fig. 9, insert). There is an over-all reticular pattern of alternating greater and lesser electron-opaque areas. Areas of greater electron-opacity seem to be interconnected and form a pattern of repeating alterations, varying in width from approximately 15 to 40 A. In many areas these latter structures surround less electron-opaque areas, forming a unit of about 100 A in diameter (Fig. 9, insert).
**Figure 2** Zone of articular cartilage. Cells in articular cartilage resemble fibroblasts. The Golgi area (GA) is well defined, and dilated endoplasmic reticulum (ER) is present. Matrix is composed of well-ordered bundles of collagen fibers (CF). Thin filamentous material (F) can be seen between bundles. × 10,000.

**Figure 3** Zone of epiphyseal cartilage. Cells in this area are chondroblasts. They are generally round, and have a scalloped cytoplasmic border and dense cytoplasm. A lacunar space (L) is present. Matrix (M) is composed of randomly distributed thin fibers and small granules. × 10,000.
**FIGURE 4** Zone of hypertrophic cells. Cells from this area are larger than those seen in epiphyseal cartilage and show an absence of well-defined cell organelles. A hypertrophic Golgi area (GA) characterized by large vacuoles is present. The lacunar space (L) is very prominent and the matrix (M) contains thin fibers and granules larger than those seen in the zone of epiphyseal cartilage. × 10,000.
The appearance of granules in the matrix is similar with all fixatives. When aldehyde fixation is used or CPC is added to the fixative, dense granules, similar to those seen in matrix, are visible on the cell membrane and in the lacunar space (Fig. 10). These granules are rarely seen in the latter regions when osmium tetroxide is used alone as the fixative. The presence of granules in lacunae and on cell membranes is more marked in the zone of flattened cells than in the zone of hypertrophic cells. Glutaraldehyde-fixed material from the zone of epiphyseal cartilage was not examined.

**Histochemical**

**Enzyme Digestion:** Both hyaluronidase and trypsin digestions cause a decrease in size and number of granules in the matrix (Fig. 11). Granules on cell membranes and those found free in the lacunar space in aldehyde-fixed tissue are missing or reduced in amount in sections from blocks subjected to enzyme digestion. In hyaluronidase-treated blocks much smaller dense particles, which were interpreted as possible remnants of matrix granules, are scattered throughout the matrix. There is no apparent change in appearance of fibers following treatment with either enzyme. The cytoplasmic contents of the cells are markedly reduced following trypsin digestion but not following hyaluronidase. Sections from control blocks incubated in buffer and in inactivated enzyme are similar to sections from blocks fixed with aldehyde. When stained with toluidine blue, there is a marked loss of metachromasia in sections from digested blocks as compared to similar sections from control blocks.

**Colloidal Iron:** There is a preferential deposition of colloidal iron on granules rather than on fibers (Fig. 12). The granules of the matrix, the granules on the cell surface, and the granules in the lacunae of aldehyde-preserved tissue are heavily stained. There are some iron particles in the background but, when sections are counterstained with uranyl acetate, no consistent relation between these particles and fibers can be identified. Staining of hyaluronidase-treated blocks with colloidal iron reveals a decrease in iron deposition concomitant with the decrease in granules.

**Chemistry**

The amount of hexosamine and the uptake of radiosulfate per milligrams of dry weight are shown in Table I. There was a significantly greater amount of hexosamine and radiosulfate uptake in the zone of hypertrophic cells than in the epiphyseal area.

Fig. 13 shows the amount of hexosamine eluted as acid mucopolysaccharide CPC complexes from the microcolumns. The 1% CPC fraction which contains hexosamines from several sources, including glycoproteins, acid mucopolysaccharides, and the papain digestion mixture itself, is not shown in the graph. Of the other fractions, those eluted with 0.4 and 0.5 MgCl₂ show striking differences between the epiphysis and zone of hypertrophic cells. These fractions from the zone of hypertrophic cells contain much more hexosamine than those from the epiphyses. The uptake of radioactivity follows the same pattern, with much more radioactivity, in the 0.4 and 0.5 M eluates from the zone of hypertrophic cells than in corresponding eluates from the epiphyseal cartilage.

**Discussion**

In previously reported electron microscopic investigations of cartilage, matrix fibers, amorphous background material (13, 15, 40, 44, 50), and oc-
FIGURE 8 Zone of epiphyseal cartilage. Higher magnification of matrix in Fig. 3. Fibers (F) measure about 200 A in width. Dense granules (G) can be seen both associated with fibers and apparently lying free in matrix. X 60,000.

FIGURE 9 Zone of hypertrophic cells. Higher magnification of matrix seen in Fig. 4. Granules (G) are larger (300–700 A) and denser than in the zone of epiphyseal cartilage (see Fig. 8). Some granules are closely associated with fibers, and many have small fibrillar projections (FP) which have staining characteristics similar to those of fibers. An accumulation of granules can be seen at the interface (I) of the lacunar space and matrix. X 60,000.

Insert. Higher magnification of one of the granules. The substructure appears to be an electron-opaque reticulum (R) varying in width from 15 to 40 A. In some areas these structures form circular units about 100 A in diameter (CU). X 840,000.
FIGURE 10  Zone of flattened cells. Section from a block fixed in glutaraldehyde and osmium tetroxide. Dense granules (G) are seen on the surface of the cells and in the lacunar space. The material in vacuoles (v) is composed of similar but smaller granules and fibrils. X 15,000.

Casionally dense matrix granules (13, 38, 40, 48) have been described. In these previous reports it has been suggested that the granules represent mucoprotein (38), a canalicular system (2), or mineral deposits (13).

Chemically, cartilage has been shown to be composed of collagen (32), non-collagenous protein (33), acidic mucopolysaccharides, which are predominantly chondroitin sulfates A and C (25, 27), and other carbohydrates (35). It has been demonstrated that chondroitin sulfate is consistently associated with protein (26, 30, 46).

By ultrastructural histochemical techniques, Revel (37) has demonstrated the extracellular location of protein-polysaccharide in cartilage matrix. However, there have been no corresponding studies correlating previously described structures, seen in electron micrographs of cartilage matrix, with the distribution of protein polysaccharide, as seen by electron microscopic histochemical techniques. In the present study, results of adapting colloidal iron staining and hyaluronidase digestion to electron microscopy indicate that the dense granules are at least one structural representation of protein-polysaccharide within cartilage matrix.

The preferential deposition of colloidal iron on granules indicates that they contain acid mucopolysaccharide. The specificity of the Hales' iron method has been questioned, but recently several authors have modified its use (29) and now consider it among the most specific stains available.
FIGURE 11 Section from a block incubated with hyaluronidase. There is a decrease in size and number of granules, with no noticeable effect on fibers (F). There are small particles (P) scattered throughout the matrix which may be remnants of granules. X 60,000.

FIGURE 12 Colloidal iron. Granules (G) are heavily impregnated with small colloidal iron particles, while fibers (F) show little affinity for this material. X 60,000.

for demonstrating sulfated mucopolysaccharides (11). The basis of this specificity is the use of a colloidal iron solution with a pH of approximately 2. It is proposed that, at this pH, ionization of weakly acidic radicals will be suppressed so that only sulfate or carboxyl groups will react with positively charged iron particles. When this technique was used, granules were the only component of cartilage that selectively bound colloidal iron. No preferential uptake of colloidal iron was found on fibers, and digestion with hyaluronidase did not alter the slight amount of homogenous background iron. The presence of a background of iron particles after hyaluronidase digestion suggests random precipitation rather than staining of an amorphous acid mucopolysaccharide component.

The granules are removed by testicular hyaluronidase, an enzyme that has been shown by
TABLE I

<table>
<thead>
<tr>
<th>Source of sample</th>
<th>Hexosamine (μg/mg dry wt)</th>
<th>Radiosulfate (cpm/mg dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epiphysis</td>
<td>16 78 ± 16</td>
<td>4.7 ± 1.6</td>
</tr>
<tr>
<td>Zone of hypertrophic cells</td>
<td>15 134 ± 28</td>
<td>11.0 ± 4.5</td>
</tr>
</tbody>
</table>

Meyer to be relatively specific for chondroitin sulfates A and C and hyaluronic acid (24). Since chemical studies have not shown hyaluronic acid to be present in cartilage matrix (25, 27), the action of this enzyme in the present studies is presumably upon chondroitin sulfate. The small, dense particles remaining after digestion with this enzyme may possibly represent residual protein. The fact that the granules were removed by trypsin indicates that a protein component is also present and that the stability of the complex depends on the integrity of both protein and polysaccharide. It has been demonstrated previously that proteolytic enzymes will destroy the protein-polysaccharide complex in vivo (47, 49, 51).

Note should also be taken of the correlation between the size and number of granules in the zones of both epiphyseal cartilage and hypertrophic cells and the results of chemical and radioisotope data. It has been demonstrated previously that inorganic sulfate is taken up very rapidly by cartilage and immediately incorporated into chondroitin sulfates A and C (7, 8, 9). Studies of radiosulfate distribution, after microdissection of cartilage into zones, consistently showed that there was greater activity associated with the zone of flattened and hypertrophic cells than associated with that of the epiphysis. There was also an increase in total hexosamine in the zone of hypertrophic cells compared with the epiphysis. Differential solubility studies of CPC acid mucopolysaccharide complexes indicated that the increase in hexosamine was due to chondroitin sulfate. This conclusion is based on the work of Scott (45) who has shown that chondroitin sulfates A and C are eluted from columns in the range between 0.4 and 0.7 M MgCl₂ in this system. In the present work the most striking differences were noted in the 0.4 M MgCl₂ fraction. An ultrastructural comparison of the two zones (Fig. 8 and 9) at comparable magnifications shows a marked increase in size of granules in the hypertrophic zone compared with that in the epiphysis, thus correlating directly both with chemical determination of acidic mucopolysaccharide and with distribution of radioactive sulfate in these tissues. This is the pattern that would be predicted if the hypothesis that granules contain protein-polysaccharide is correct.

Revel (37) has performed similar electron microscopic histochemical experiments on cartilage from mice and salamanders; he used colloidal thorium (thorotrast) rather than colloidal iron for staining acid mucopolysaccharides. Dense accumulations of thorium in roughly circular patterns about 0.2-0.4 μm in diameter were seen at low magnifications. At higher magnifications, areas of central density surrounded by halos of less dense material could be seen. No attempt was made to

![Graph](image-url)
correlate the pattern of thorium staining with previously described structures of cartilage matrix. In the present study it may be that the smaller size of colloidal iron offers less contrast than thorium at low magnifications but greater resolution than thorium at higher magnifications, allowing correlation of staining with granules. The localization of colloidal iron to matrix granules as seen in this study, in contrast to the more diffuse deposition of thorium seen by Revel, may be due to differences in stains (colloidal iron vs. thorium), embedding material (epon vs. methacrylate), or fixation (glutaraldehyde-osmium tetroxide vs. osmium tetroxide). The possibility also exists that there is a species difference in the distribution of protein-polysaccharide in cartilage matrix. It is probable that colloidal iron does not penetrate epon very well (5) and that staining seen in the present study represents reaction with exposed surface groups. If this is the case the unstained areas seen between granules still may contain acidic mucopolysaccharide but in insufficient amounts to concentrate colloidal iron particles over background density. Thus these experiments do not entirely preclude the existence of protein-polysaccharide in other areas of cartilage matrix but do indicate that granules contain focal accumulations of this material.

Previous chemical work has suggested a close relationship between protein-polysaccharide and collagen (3, 14, 22). In the present experiments there is further evidence that this may be the case, for many granules are closely associated with fibers. In addition, many granules contain small projections which, after staining with heavy metals, show contrast similar to that of fibers. The nature of these small projections was not determined, but it has been suggested (38) that they represent monomeric collagen. If this is the case, granules may represent a mixture of immature collagen and protein-polysaccharide from which polymerization of collagen into mature matrix fibers may occur.

The substructure of granules is reasonably consistent with structural models that have been proposed for the protein-polysaccharide complex of cartilage matrix. Mathews and Lozaityte (23) found the macromolecular unit of this complex to have a molecular weight of $4 \times 10^6$ and a proposed length of 3700 A. This unit was thought to be composed of chondroitin sulfate chains extending perpendicularly from a linear, central protein core. Since the degree of ionization of sulfate groups would influence extension of the chondroitin sulfate chains, the diameter of the molecule would depend upon the ionic environment. Based on the conditions of their experiments, an approximate diameter of 37 A was calculated. Later work by Partridge (34) was also in good agreement.

The observations, in this study, of electron-opaque areas approximately 15-40 A in width within the granules is reasonably consistent with the diameter of the models of the protein-polysaccharide complex proposed by Mathews and Lozaityte (23) and by Partridge (34). Variation in width of dense areas within granules found in this study, and discrepancy between these widths and those proposed above, may be explained by fixation artifact or oblique sections of linear material. No estimate of length is possible by the techniques of the present study.

There is some evidence that the regularly repeating alterations in width of electron-opaque areas may not be artifact but may represent interconnected particles forming linear structures. Jackson (16) extracted protein-polysaccharide from cartilage by the method of Maliwista and Schubert (19) and examined the 20 S fraction of Gerber (12) by electron microscopy using negative staining techniques. Electron micrographs of this fraction showed subunits measuring $45 \times 55$ A, which were proposed as the protein core of the complex. In some preparations Jackson found filaments and suggested that the subunits were aggregated into linear arrays to form protein-polysaccharide filaments in vivo. Whether this hypothesis could account for variations in width of the electron-opaque areas in the present study, could not be determined. In addition, Jackson (16) found that in many areas these particles were arranged in rings of 5-6 units around a clear central area. We have found the same pattern in the present study (Fig. 9 insert), but the meaning of this observation is not clear.

Schubert has indicated that both hyaluronate and protein-polysaccharide occupy a large domain in solution (42). It has also been suggested that entanglement of diffuse macromolecules of protein-polysaccharide with collagen might account for some of the physico-chemical properties of cartilage (43). It is thus conceivable that granules seen by electron microscopy in the present study may represent clumping by fixation of protein polysaccharide chains which in vivo extend into the clear unstained spaces between granules and fibers.
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