In vitro chondrogenesis in mouse limb mesenchymal cells; changes in ultrastructure and proteoglycan synthesis

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SUMMARY

Mouse chondrocytes were allowed to differentiate from 11-day dissociated limb-bud mesenchymal cells cultured at high density. Over a period of 5 days in culture these cells differentiated from a culture containing a population of mesenchymal cells and myoblasts into one containing cartilage nodules. Chondrogenesis, as demonstrated by the sulfated proteoglycans (PGS) synthesized, was monitored quantitatively and qualitatively by molecular sieve chromatography on polyacrylamide and 1% agarose columns, and by examination of cultures with phase contrast and transmission electron microscopy. Chromatographically, the PGS synthesized could be separated into three fractions (Ia, Ib, and II). In the 24 h cultures a small amount of PGS was synthesized and was predominantly of fraction II. As time in culture elapsed there was, concomitant with chondrogenesis, an increase in PGS synthesis with a preferential increase in peaks Ia and Ib. Peaks Ia and Ib were shown to have an aggregate-subunit relationship. Fraction Ia could be dissociated to chromatograph at the Ib position, but the disaggregation was not reversible. Digestion of the various chromatographic fractions extracted from the cell layer of the 5-day cultures by chondroitinase ABC and AC revealed that fraction I contained 80% chondroitin-4-sulfate, 20% chondroitin-6-sulfate, and negligible amounts of dermatan sulfate, while chondroitinase ABC and AC digestion of fraction II showed 55% chondroitin-4-sulfate, 0-37% chondroitin-6-sulfate, and 20% dermatan sulfate. Cytological studies revealed that as nodule development progressed there was a concurrent development of the endoplasmic reticulum and extracellular matrix, and a reduction in mitochondria.

INTRODUCTION

Most of the present knowledge concerning chondrogenesis in vitro has been obtained from studies on the differentiation of the somites or of limb-bud mesenchymal cells from the chick embryo. Electron microscopic studies and biochemical studies have established a series of parameters which characterize chondrogenesis. The pattern of development demonstrated by these studies shows ultrastructural changes in the closely packed mesenchymal cells which gradually differentiate into chondrocytes. These cells develop an extensive endoplasmic reticulum system, and secrete an extracellular matrix consisting of sulfated proteoglycans (PGS) and collagen, which separates the cells from one another.

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Biochemical changes are characterized by the increased synthesis of PGS and the appearance of type II collagen (Matukas, Panner & Orbison, 1967; Caplan, 1970; Searls, Hilder & Mirow, 1972; Minor, 1973; Goetinck, Pennypacker & Royal, 1974; Levitt & Dorfman, 1974; Goel & Jurand, 1975; Levitt, Ho & Dorfman, 1975; Oegenja, Hascall & Dziewiatkowski, 1975).

Very little information is available on chondrogenesis in mammalian systems. Cooper (1965) has investigated the cartilage differentiation in the somite of the developing mouse embryo, and Elmer & Selleck (1975) have presented evidence with mouse limb-buds that, with chondrogenesis, collagen secretion becomes predominantly of the cartilage type. The purpose of this study was to characterize chondrogenesis in the mouse in terms of ultrastructural changes and PGS synthesized.

MATERIALS AND METHODS

Mice used in this study were either Swiss albino outbred CD 1 mice purchased from Charles River Breeding Farm, Wilmington, Mass., or C57BL/10 and representatives from several inbred strains and their hybrids obtained from the colony maintained in the Department of Animal Genetics, University of Connecticut, through the courtesy of Dr L. J. Pierro. Trypsin was from Nutritional Biochemical Corp., Cleveland, Ohio; Minimal Essential Medium (MEM), fetal calf serum (FCS) and horse serum (HS) from Grand Island Biological Co. (GIBCO), Grand Island, New York; and the tissue culture dishes were from Falcon (no. 3001). Guanidinium hydrochloride (GuHCl) and cesium chloride (CsCl) were obtained from Schwartz-Mann, Orangeburg, New York; Triton X-100 was from Rohm and Haas, Philadelphia, Pa.; 2-(N-Morpholino) ethanesulfonic acid (MES) was obtained from Calbiochem, La Jolla, Ca; Polyacrylamide P-300 gel and agarose A-150 were both obtained from Bio-Rad, Richmond, Ca. Controlled pore glass (CPG) was obtained from Electro-Nucleonics Inc., Fairfield, N.J. The scintillation fluid used was prepared by mixing one part Triton X-100 with two parts toluene (Target Chemicals) containing 4 g/liter 2,5-diphenyloxazole (Scintillation grade), (PPO), and 0.1 g/liter 1,4-bis [2(5-Phenylloxazolyl)] benzene (Scintillation grade), (POPOP), both from Amersham Searle, Arlington Heights, II. Na$_2^{35}$SO$_4$ was obtained from New England Nuclear, Boston, Mass. Chondroitinase AC and ABC and disaccharides, 2-acetamido-2-deoxy-3-O-(β-D-Gluco-4-enepyrano-syluronic acid)-4-0-sulfo-D-galactose (Δ-Di-4S), 2-acetamido-2-deoxy-3-O-(β-D-Gluco-4-enepyra-no-syluronic acid)-6-0-sulfo-D-galactose (Δ-Di-6S) were all obtained from Miles Laboratories, Kankakee, Il. Hyaluronic acid was obtained from Biotics, Boston, Mass.

Cell cultures

Cells were derived from limb-buds obtained from 11-day Swiss albino outbred CD 1 or C57BL/10 embryos. These embryos are comparable to stage-24 chick embryos. No differences in chondrogenic development between these strains
Chondrogenesis in mouse limb mesenchymal cells

were observed. The procedure for culturing mouse limb-bud cells was the same as that used for chick limb-bud cells (Caplan, 1970; Goetinck et al. 1974). In brief, limb-buds were removed, dissociated in 2% trypsin, and a single cell suspension of cells in MEM supplemented with 3% FCS, 5% chick embryo extract, and 7% HS was made. The initial plating density of the cells was 3 x 10^6 cells/35 mm dish. At this density the entire bottom of the dish was covered with cells, and after 5 days in culture, cartilage nodules were observed.

Labeling and extraction of proteoglycan

Cultures were exposed to 100 μCi Na_2^35SO_4 for 4 h, at 24 h, 48 h, and 5 days in culture. The medium was collected, the cells were washed with phosphate buffered saline (PBS), and the wash was pooled with the medium. The cell layer was treated with a solution of 4 M-GuHCl with 1% Triton-X buffered with MES at pH 5-8. After a 24 h extraction at 4 °C, the plates were scraped clear and washed with 0.5 M-NaCl. The cell extract was pooled with the wash and dialyzed twice against 7 l. of 0.5 M-NaCl for 24 h, at 4 °C and centrifuged at 15000 rpm in a Beckman 40 rotor for 30 min.

Proteoglycan analysis

Proteoglycan was analyzed by molecular sieve chromatography using either polyacrylamide (Bio Gel P-300), 1% agarose (Bio Gel A-150 m), or controlled pore glass (CPG) as column chromatographic material. All sulfate-labeled macromolecules were found in the void volume of either Bio Gel P-300 or CPG 240 columns (Lever & Goetinck, 1976). These two materials were used to quantify the amounts of PGS synthesized, or for initial extraction of the proteoglycan to be qualitatively analyzed. The extracts of proteoglycan can be separated into three chromatographic fractions on 1% agarose columns. These fractions are identified in the order of elution as Ia, Ib, and II, with Ia being excluded from the gel (Goetinck et al. 1974). Ia is also excluded on CPG 2500 (Lever & Goetinck, 1976). In the chick, the proteoglycan found in fraction Ia has been shown to be an aggregate of that found in Ib (Goetinck et al. 1974; Lever & Goetinck, 1976).

Cell extracts and media were first analyzed on P-300 columns for the purposes of obtaining an estimate of the total amounts of proteoglycan found in them and the ratio between the two. Samples were brought up to a known volume, from which a 1 ml aliquot was applied to the column. Thirty 1 ml fractions were collected, and the entire sample was counted. Isolation of the proteoglycan of the remainder of the samples was again carried out on a P-300 column and the void volume material was then analyzed on 1% agarose in order to determine the distribution of PGS in the various chromatographic fractions. Fifty 1 ml fractions were collected.

Fraction Ia, derived from the cell layer of the 5-day culture and obtained
from a CPG 2500 column, was dissociated from its linking complex by centrifuging it on a CsCl gradient (initial density of 1.50 g/ml) in the presence of 4 M-GuHCl, buffered at 5-8 with MES. After centrifugation for 48 h at 36000 rpm in a 50 Ti rotor, the gradient was cut into three parts. The dissociated proteoglycan was found in the bottom third of the gradient.

**Glycosaminoglycan analysis**

The amounts of chondroitin-4-sulfate (Ch-4-S), chondroitin-6-sulfate (Ch-6-S) and dermatan sulfate (DS) derived from the proteoglycan of the 5-day cell layer were estimated using chondroitinase AC and ABC according to the procedure of Yamagata, Saito, Habuchi & Suzuki (1968). Carrier disaccharides were added to the digests which were chromatographed on Whatman no. 1 filter paper with 1-butanol, glacial acetic acid, and 1 N ammonia as solvents (2:3:1). Carrier disaccharides were visualized with an ultraviolet light. Strips of 0.5 cm by 2.5 cm were cut and quantified by liquid scintillation spectrometry.

**Ultrastructure**

Cultures from 24 h, 48 h, and 5 days were fixed for 6 h in 2 % glutaraldehyde, post-fixed in 2 % OsO₄, stained with uranyl acetate for 1 h, quickly dehydrated, and embedded in Epon 812. The Epon with embedded cells was then peeled off the culture dish and glued flat onto an epon block. Thin sections (silver to gold) were cut, stained again with uranyl acetate and lead citrate, and examined with a Philips electron microscope at 80 kV.

**Alphabetical order of abbreviations**

Ch-4-S, chondroitin-4-sulfate; Ch-6-S, chondroitin-6-sulfate; CPG, controlled pore glass; cpm, counts per minute; CsCl, cesium chloride; DS, dermatan sulfate; ER, endoplasmic reticulum; FCS, fetal calf serum; GuHCl, guanidinium hydrochloride; HA, hyaluronic acid; HS, horse serum; KS, keratan sulfate; MEM, Minimal Essential Medium; MES, 2-(\[\text{N}]-Morpholino)-ethanesulfonic acid; PBS, phosphate buffered saline; PGS, sulfated proteoglycans; TEM, transmission electron microscope.

**RESULTS**

**Histology**

Phase contrast microscopic observations of the cultures at 24 h, 48 h, and 5 days reveal the transformation of the unorganized mesenchymal cells into cultures showing maximal nodular development at 5 days (Fig. 1A–C). At 24 h, the cultures are characterized by homogeneous mesenchymal cells interspersed with some apparent myoblasts (Fig. 1A). At 48 h in culture, the beginning of some organization, identified as early nodular swirls, can be seen (Fig. 1B). In both the 24 h and 48 h cultures, the cells appear relatively larger than at later stages. However, by 48 h in culture, the cells are also distinguished
Fig. 1. A, B, C, Phase microscopy of cultured mouse limb-buds at 24 h (A), 48 h (B), and 5 days (C) in culture. × 320.
Fig. 2. Mesenchymal cells at 24 h in culture. × 7700.

Fig. 3. Mitochondria present in the 24 h cultured cells. × 33275.
Fig. 4. Mesenchymal cells at 48 h in culture. × 7700.

Fig. 5. Mesenchymal cells at 48 h in culture in an area of early nodule formation. × 5600.
by a dark granular substance in the area of early nodular formation. Five-day cultures show profuse numbers of well established nodules embedded in an iridescent matrix, which binds Alcian green. This stain binds to the polyanions in the matrix characteristic of cartilage (Ham & Sattler, 1968). Maximal cartilage development is observed in the 5-day culture (Fig. 1C).

Transmission electron microscopic (TEM) studies reveal in greater detail the ultrastructural development and transformation of the cells into chondrocyte nodules. Figures 2 and 3 show the morphological appearance of the cultured cells at 24 h of development. These cultures are distinguished by having closely packed, large cells, with large nuclei, as seen in Fig. 2. Occasionally, small amounts of fibrillar extracellular material thought to be collagen is visible (Fig. 2, arrow). At higher magnification, one can see in the cytoplasm a large number of mitochondria, many unbound polysomes, and an underdeveloped endoplasmic reticular (ER) system (Fig. 3).

By 48 h in culture the cell morphology has become heterogeneous. A majority of these cells are still large, round, tightly packed, and appear similar to those seen at 24 h (Fig. 4). However, in areas of early nodular development, the cells appear slightly more elongated than in the 24 h culture, and the ER system appears more extensive (Fig. 5). At higher magnification, one can see in these cells polysomes lining the walls of the ER system (Fig. 6). Figure 7 shows a large Golgi complex suggesting the beginning of secretory function as well as a large mitochondrion, characteristic of the 24 h culture.

At 5 days in culture full chondrogenic expression is seen (Fig. 8). This is indicated by the presence of collagen fibrils and proteoglycan granules in the extracellular matrix (Fig. 8). Cells located within the nodule contain mitochondria, although they appear smaller and fewer in number (Fig. 8). The ER system has become well established and contains many vacuoles, which is characteristic of chondrocytes. When these cultured cells are compared to femoral chondrocytes, fixed in situ, from newborn mice (Fig. 10), similarities are evident; the development of the ER, the scalloped edges of the cells, the reduced number of mitochondria, and the presence of a well established extracellular matrix all appear similar. Differences between in vitro and in vivo preparations are also apparent. The cultured cells are closer to each other than in the in vivo preparations, and there is a slight reduction in the extracellular matrix. This could be a consequence of differences in chronological development, or of stress imposed upon the cells during culture, or during the fixation process.

In the 5-day culture, cells located outside of the nodule appear round and they are closer together. The ER system here is still fairly well established, although a large number of mitochondria are still evident (Fig. 9).

**Biochemistry**

The total amounts of PGS synthesized were estimated by measuring the amounts of Na₂³⁵SO₄ incorporated into macromolecules during a 4 h exposure
Fig. 6. Endoplasmic reticulum at 48 h in culture. × 54395.

Fig. 7. Golgi complex at 48 h in culture. × 37760.
Fig. 8. 5-day cultured cell in area outside the nodule. $\times 30250$.
Fig. 9. 5-day cultured cells inside the nodule. $\times 19650$. 

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ending at 24 h, 48 h, and 5 days. The results of this analysis are given in Table 1. They are presented in terms of total PGS associated with the cell layer and that found in the medium. Two major changes can be seen. First, there is a 20-fold increase in total sulfate incorporated over the 5-day period; and second within
Table 2. Distribution of PGS between peaks Ia, Ib and II in cell and medium analyzed on 1% agarose*

<table>
<thead>
<tr>
<th>Stage</th>
<th>Ia</th>
<th>Ib</th>
<th>Ia+Ib</th>
<th>II</th>
<th>Stage</th>
<th>Ia</th>
<th>Ib</th>
<th>Ia+Ib</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>5-1</td>
<td>10-4</td>
<td>15-5</td>
<td>84-3</td>
<td>24 h</td>
<td>5-1</td>
<td>10-4</td>
<td>15-5</td>
<td>84-3</td>
</tr>
<tr>
<td>48 h</td>
<td>3-6</td>
<td>7-4</td>
<td>11-0</td>
<td>89-0</td>
<td>48 h</td>
<td>3-6</td>
<td>7-4</td>
<td>11-0</td>
<td>89-0</td>
</tr>
<tr>
<td>5-day</td>
<td>8-4</td>
<td>22-4</td>
<td>30-8</td>
<td>69-1</td>
<td>5-day</td>
<td>8-4</td>
<td>22-4</td>
<td>30-8</td>
<td>69-1</td>
</tr>
</tbody>
</table>

* Expressed in terms of % total cpm recovered from 1 % agarose column.

this 20-fold increase, there is a shift in the amount of PGS which remains associated with the cell from 29 % at 24 h, to 52 % at 48 h, and 61 % at 5 days. The combination of these temporal changes results in a 40-fold increase of PGS which remains associated with the cell layer and an 11-fold increase of PGS found in the medium.

The proportions of PGS found in peak Ia, Ib, and II by chromatography on 1% agarose are given in Table 2. The data are presented for the PGS found in the cell layers and in the media for the time periods under investigation. In the cell layer at 24 h, 23-5 % of the total PGS was found in peak Ia and Ib, and 76 % in peak II. By 48 h in culture 40 % was found in peaks Ia and Ib, and 60 % in peak II. At 5 days in culture 85 % was found as peaks Ia and Ib material and 15 % as peak II. In contrast to the cells, the composition of the medium at 24 h was composed of 15-5 % Ia and Ib, and 84 % peak II material; by 48 h the make-up of the PGS was 10 % Ia and Ib, 90 % peak II, and at 5 days 30 % was found to be Ia and Ib material with 70 % peak II. The higher incorporation of Na$_2$^{35}$SO$_4$ into PGS associated with the cell, relative to the medium (Table 1), and the shift of the predominant molecular species from peak II material at 24 h to peak I material at 5 days (Table 2), indicates that chondrogenesis is taking place.

The distribution of Ch-4-S, Ch-6-S and DS in the various molecular species derived from the 5-day cell layer was determined with chondroitinase AC and ABC digestion. With chondroitinase ABC, DS is digested and migrates with Ch-4-S. The difference between the total cpm found at the Ch-4-S position, using chondroitinase ABC and AC, constitutes the amount of DS present in the sample. The results of the digestion are given in Tables 3 and 4. They are expressed in terms of net cpm and the % of total counts at each position. Peak Ia and Ib show identical patterns of digestion. They both consist of 80 % Ch-4-S, 20 % Ch-6-S, and negligible amounts of DS. Peak II, on the other hand, contains 55 % Ch-4-S, 3 % Ch-6-S, 20 % DS, and 20 % chondroitinase ABC resistant material.

Dissociation of the Ia molecule with 4 M-GuHCl and separation from its
Table 3. Chondroitinase AC and ABC digestion of the various A-150 fractions extracted from the 5-day cell layer*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Origin</th>
<th>Ch-6-S</th>
<th>Ch-4-S</th>
<th>Total Net cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>0</td>
<td>280</td>
<td>1230</td>
<td>1510</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.5%</td>
<td>81.5%</td>
<td></td>
</tr>
<tr>
<td>ABC</td>
<td>0</td>
<td>165</td>
<td>1170</td>
<td>1335</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.4%</td>
<td>87.6%</td>
<td></td>
</tr>
<tr>
<td>Ib</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>230</td>
<td>915</td>
<td>5030</td>
<td>6175</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.7%</td>
<td>81.5%</td>
<td></td>
</tr>
<tr>
<td>ABC</td>
<td>185</td>
<td>895</td>
<td>4195</td>
<td>5275</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.5%</td>
<td>79.5%</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>590</td>
<td>0</td>
<td>725</td>
<td>1315</td>
</tr>
<tr>
<td></td>
<td>44.9%</td>
<td></td>
<td>55.1%</td>
<td></td>
</tr>
<tr>
<td>ABC</td>
<td>330</td>
<td>60</td>
<td>1195</td>
<td>1585</td>
</tr>
<tr>
<td></td>
<td>20.8%</td>
<td>3.8%</td>
<td>75.4%</td>
<td></td>
</tr>
</tbody>
</table>

* Data are presented as net cpm recovered and as % of total net cpm in each digestion.

Table 4. Compositional analysis of the glycosaminoglycan derived from the data presented in Table 3

<table>
<thead>
<tr>
<th>Fraction</th>
<th>DS</th>
<th>Ch-6-S</th>
<th>Ch-4-S</th>
<th>ABC resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>0</td>
<td>15.45%</td>
<td>84.55%</td>
<td>0</td>
</tr>
<tr>
<td>Ib</td>
<td>0</td>
<td>15.90%</td>
<td>80.50%</td>
<td>3.60%</td>
</tr>
<tr>
<td>II</td>
<td>20-30%*</td>
<td>3.8%</td>
<td>55.10%</td>
<td>20.80%</td>
</tr>
</tbody>
</table>

* Amounts of DS were estimated by subtracting the % of cpm found in the Ch-4-S position after AC digestion from those found after ABC digestion.

linking complex by CsCl gradient centrifugation, resulted in a disaggregated form (Ib) as demonstrated by molecular sieve chromatography on a CPG 2500 column. Heinegård & Hascall (1974) have presented a model of the monomeric and aggregated form of PGS. In this, they show aggregation of the monomer to be an assembly of PGS onto hyaluronic acid (HA), which is finally stabilized by a link protein. Attempt to reaggregate the mouse PGS Ib with either its own linking complex, or that derived from chick chondrocytes, or pure hyaluronic acid were unsuccessful.
In vitro chondrogenesis in the mouse has been characterized by measuring the increased $\text{Na}_2\text{SO}_4$ incorporation into PGS which occurs simultaneously with ultrastructural cell changes and the appearance of proteoglycan granules and collagen fibrils in the extracellular matrix.

The nodule formation characterized by phase microscopic studies shows a gradual transformation of mesenchymal cells, at 24 h, into early nodular swirls at 48 h, and finally into profuse iridescent nodules dispersed in apparently undifferentiated cells, at 5 days. These nodules stain an intense green with Alcian green which is specific for polyanions such as PGS. The nodules obtained in the mouse limb-bud cultures described in this study more closely resemble those obtained with the chick limb-bud (Caplan, 1970; Goetinck et al. 1974) than the mouse limb-bud cultures presented by Elmer & Selleck (1975).

Ultrastructural changes occurring over this period of differentiation consist in the development of an extensive ER, the establishment in the extracellular matrix of proteoglycan granules and collagen, and a reduction in the number of mitochondria. Levitt et al. (1975) have presented transmission electron microscopic (TEM) studies on chick limb-bud cells in culture. The morphological changes seen as cells differentiate into chondrocytes appear to be similar in both species. These authors suggest that the changes seen in the mitochondria are of a degenerative nature, and that proceeding concomitantly with chondrogenesis, the cells become more dependent on anaerobic respiration (Levitt & Dorfman, 1974). The development of the endoplasmic reticulum and Golgi complex seen in the 48-h and 5-day cultures and in in vivo preparations are extensive and appear consistent with secretory function.

Biochemical changes over the 5-day culture period also follow the parameters for chondrogenic identification. This is typified by an increase in total sulfate incorporation into PGS, coupled with a preferential increase in fraction I PGS. The analysis on 1 % agarose shows that 23 % of the PGS associated with the cell at 24 h is in fraction I. In the 5-day cultures 85 % of the cell-associated PGS is found in fraction I while in the medium only 30% of the PGS is peak I material. Along with this shift, a 40-fold increase was found in the cell-associated extracts. Therefore, in addition to the increased total synthesis of PGS and the preferential increased synthesis of peak I material, the majority of the PGS remains associated with the cell layer, resulting in the establishment of matrix which was revealed with the histological studies. Of the total increase in PGS synthesized, the major portion of this increase took place during the first 48 h in culture, and to a lesser degree between the 48 h and the 5-day period.

An interesting observation can be seen in both the 48-h TEM pictures and 48-h 1% agarose chromatographic analysis. At this stage the TEM pictures reveal that the endoplasmic reticular system is already becoming organized and Golgi complexes are developed. It is also at this stage that a sizable amount of
fraction I PGS is first seen (Table 2). The majority of this is cell-associated (Table 1), while the medium still shows predominantly peak II PGS. This suggests a transitory period from a non-secretory to a secretory cell. Evidence has been presented for a similar molecular and histological sequence of events leading to chondrogenesis in the chick limb-bud system (Caplan, 1970; Goetinck et al. 1974; Levitt et al. 1975).

Levitt & Dorfman (1974) have presented evidence showing PGS I material to be present in precartilage stage-24 limb-bud cultures. Goetinck et al. (1974) have shown that PGS I material is also present in stage-18 limb-buds in vivo. This evidence suggests that the sudden surge in synthesis of peak I material is a regulatory phenomenon uniquely found in chondrocytes. Pennypacker & Goetinck (1976) have re-enforced this hypothesis in their comparison of normal and nanomelic chondrocytes. Mutant nanomelic chick chondrocytes produce PGS at levels which are 10% of normal chondrocytes (Mathews, 1967; Palmoski & Goetinck, 1972). These reduced levels of synthesis in the mutant are reflected only in reduced amounts of PGS I. It has been shown that the nanomelic chondrocytes, like precartilaginous mesenchymal cells, produce small amounts of peak I material suggesting that the nanomelic phenotype results from a regulatory mutation (Pennypacker & Goetinck, 1976; Goetinck & Pennypacker, 1977).

Several laboratories have reported on the aggregation of the subunit (Ib) with hyaluronic acid and one, possibly two stabilizing polypeptides (Hascall & Heinegård, 1974; Heinegård & Hascall, 1974; Oegenia et al. 1975). The present molecular sieve chromatography studies revealed a striking difference between chick and mouse chondrocytes. In the chick the major molecular species is in the aggregated form (Goetinck et al. 1974). In the mouse the disaggregated form is found to be the major molecular species. Experiments presented in this paper show that upon CsCl centrifugation in the presence of 4 M-GuHCl, the 1a material shifts to the Ib position, or dissociated state. However, we were unable to reaggregate this molecule with either the fraction of the gradient which in the chick or the bovine system contains aggregating material, or with hyaluronic acid. This could be due to several reasons. Firstly, assuming that the linking system in the mouse is similar to that found in the chick and bovine system, perhaps the mouse molecule is more sensitive to the dissociation procedure than the chick, such that the linking portion of the protein core is somehow altered or destroyed. This would have to be a very small portion of the total molecule, because chromatographic studies of this material yield radioactivity only in the Ib position. If the mouse molecule is more sensitive to dissociation it might explain the abundance of Ib in the cell layer extract, since this was obtained under dissociative conditions. However, the predominance of Ib in the medium cannot be explained on the basis of sensitivity to dissociation since the medium was never exposed to dissociative conditions. Therefore, the possibility of a different linking system in the mouse does exist.
We have shown that the ontogeny of PGS in the mouse and chick is similar. This parallels the work which had demonstrated that the collagen shift from Type I to Type II also occurs in both the mouse and in the chick differentiating chondrocyte (Elmer & Selleck, 1975; Linsenmeyer, Toole & Trelstad, 1973).

Enzyme digests of peak Ia and Ib of the cell associated extract revealed 80% Ch-4-S. These results differ from those obtained for the chick where Ch-6-S is the major molecular species (Levitt & Dorfman, 1974). The predominance of Ch-4-S in the rodent was also reported by Handley & Phelps (1972), using neonatal rat epiphyseal cartilage. They reported PGS consisting of 65% Ch-4-S, 15% Ch-6-S, and 2% keratan sulfate (KS). The possibility of peak II material being a breakdown product of peak I material has never been tested directly before. The presence of 20% DS in peak II not found in peak I, seems to indicate that peak II material is a separate molecular species of PGS, which in this case could be synthesized by cells other than chondrocytes. The separate molecular status of peak II material is further supported by its relatively high content (20%) of chondroitinase ABC resistant material. In this study we do not characterize ABC resistant material although KS and heparin sulfate are known to be resistant to ABC digestion.

There was 3-7% of chondroitinase ABC resistant material in fraction Ib and none was detected in fraction Ia. This could result from the fact that the Ia fraction analyzed contained only one fourth as much radioactivity as the Ib fraction. This ABC resistant material in fraction Ib could be KS since Hascall, Oegenia, Brown & Caplan (1976) have reported that the monomer (Ib) obtained from chick limb-bud culture contains 7% KS.

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