Monomeric and aggregate proteoglycans in the chondrogenic differentiation of embryonic chick limb buds

By N. S. VASAN¹ AND J. W. LASH²

From the Department of Anatomy, College of Medicine and Dentistry of New Jersey and Department of Anatomy, University of Pennsylvania

SUMMARY

Proteoglycan heterogeneity was studied during the in vivo differentiation of embryonic chick limb cartilage. Recently, it has been shown that during the differentiation of limb cartilage the proportion of the aggregated form of proteoglycans increases whereas the un-associated monomeric forms decrease, and this has been related to the synthesis of two link proteins at a specific stage of differentiation. In this study it is suggested that the appearance of the aggregate formation is also due to synthesis of a stable hyaluronic acid binding region of the core protein. Thus, it can be concluded that differential gene expression for these proteins takes place as a differentiation phenomenon.

INTRODUCTION

Chondrogenesis in the vertebrate embryo involves an initial phase of mesenchymal cell accumulation and aggregation, followed by differentiation and the production of cartilage matrix. In this paper additional evidence is presented for the hypothesis that unassociated proteoglycan monomers are present in pre-cartilaginous stages and become, as development progresses, aggregated by the synthesis of link protein(s) and the hyaluronic acid binding region of the core proteins (Vasan & Lash, 1977a). In addition, the semantic confusion concerning a ‘cartilage specific’ proteoglycan is examined in light of recent reports (Holtzer et al. 1975; Vasan & Lash, 1977b).

Proteoglycans synthesized by chondroblasts are different from those synthesized by mature, fully expressed chondrocytes. Chondroblasts are capable of synthesizing very small quantities of proteoglycan aggregates and large amounts of unassociated proteoglycan monomers (Vasan & Lash, 1977a). The differential gene expression of chondrocytes has been correlated with the synthesis of

¹ Author’s address: Department of Anatomy, College of Medicine and Dentistry of New Jersey, 100 Bergen Street, Newark, New Jersey 07013, U.S.A
² Authors address: Department of Anatomy, School of Medicine/G3, University of Pennsylvania, Philadelphia, Pennsylvania 19104, U.S.A. (Requests for reprints.)
proteoglycan aggregates which can be identified by their exclusion from a gel column (Palmoski & Goetinck, 1972; Levitt & Dorfman, 1974) or by the sedimentation pattern in a sucrose density gradient centrifugation (Kimata, Okayama, Oohira & Suzuki, 1974; Okayama, Pacifici & Holtzer, 1976). These analyses have led to the designation of a 'cartilage specific proteoglycan'. The existence of such cartilage specific proteoglycans (which is purported to be characteristic of differentiated cartilage) has been shown to be present in precartilaginous somitic and limb-bud tissue (Goetinck, Pennypacker & Royal, 1974; Vasan & Lash, 1977b) and nanomelic chondrocytes (Pennypacker & Goetinck, 1976).

The aggregation of proteoglycan monomers requires interactions between at least three types of molecules; the proteoglycan monomer, smaller link proteins and hyaluronic acid (Hascall & Heinegård, 1975; Hardingham, Ewins & Muir, 1976). Recently, it has been shown that during the differentiation of limb cartilage the proportion of the aggregated form of proteoglycans increases, whereas the unassociated monomeric forms decrease. It has also been shown that only one link protein can be detected from precartilaginous stages of differentiation and two link proteins are detected in differentiated cartilage (Vasan & Lash, 1977b). In the present study it is shown that the monomeric proteoglycans from precartilaginous mesenchyme of limb buds (prior to stage 24–25) have little or no capacity to interact with hyaluronic acid, whereas the monomeric proteoglycans of differentiated cartilage (stage 35) are capable of interacting with hyaluronic acid. This suggests that the core protein in precartilaginous mesenchyme lacks the hyaluronic acid binding region and the genetic information for this binding region is expressed at a later stage of differentiation.

**MATERIALS AND METHODS**

Limb buds from stage 12 (stage 12 is pre-limb stage), 18, 24 and 35 were obtained when White Leghorn chick embryos and stages of development were determined according to the staging series of Hamburger & Hamilton (1951). Precartilaginous stages-12, -18 and -24 limb buds were cut free of the embryos and analyzed without further dissection. The cartilages of the later stages were dissected free of adhering tissues, and cut into small (1 mm³) pieces. The proteoglycans were labeled by incubating them in F12X medium (Marzullo & Lash, 1970) containing 20 μCi/ml of carrier-free Na₂³⁵SO₄ (Amersham/Searle, Arlington Heights, Ill., U.S.A.). After 8 h of incubation in a humidified incubator in 95% air and 5% carbon dioxide, the tissues were rinsed several times with Simms' balanced salt solution (Simms & Sanders, 1942).

Proteoglycans were extracted with 0·4 M guadininium hydrochloride (GuHCl) (Ultra pure grade, Shwartz/Mann, Orangeburg, N.Y., U.S.A.), buffered with 0·05 M sodium acetate, pH 5·8, containing 0·10 M 6-aminohexanoic acid (Aldrich Chemical Co., Milwaukee, Wisc., U.S.A.) and 0·005 M benzamidine hydrochloride (Eastman Kodak, Rochester, N.Y. U.S.A.) 10 mM EDTA to inhibit
proteolysis (Oegema, Hascall & Dziewiatkowski, 1975). The tissues were extracted in the above solvent (10–15 times the volume of the tissue) by gentle agitation on a rotary shaker for 24 h at 4 °C. The products of the 0.4 M-GuHCl extraction constitute fraction 1, which are obtained under associate conditions, as described by Heinegård (1972). At this concentration of GuHCl the aggregated proteoglycans (PGA) remain associated and the unassociated proteoglycan monomers (uPGM) are extracted. It has been shown by Lash & Vasan (1978) that 20% of the aggregates in 13-day embryonic sternal cartilage are removed under associative extraction conditions. The tissues were subjected to further extraction as above, except that GuHCl concentration was raised to 4.0 M (the dissociative condition of Heinegård, 1972). Under these conditions, the remaining proteoglycan aggregates are dissociated and extracted as monomeric proteoglycans (PGM). This second extract, which represents primarily proteoglycans which were in the aggregated form, is termed fraction 2. Both fractions were dialysed against 0.04 M sodium sulfate for 12 h at 4 °C, followed by 48 h against cold distilled water. This procedure causes the proteoglycans in the 4.0 M-GuHCl extract to re-aggregate. The fractions were centrifuged at 20850 g for 30 min and the superatants were lyophilized. The resulting material was dissolved in 0.5 ml of deionized water and a 0.1 ml of aliquot was used for determining incorporation of [35S]sulfate in an Intertechnique 4200 liquid scintillation counter. The samples were dissolved in toluene containing 6.0 g of PPO/liter (2,5-diphenyloxazole), 0.3 g of POPOP/liter (1,4 bis-(5-phenyloxazol-2yl)benzene) (Packard Instruments, Downers Grove, Ill., U.S.A.) and 5% (v/v) Biosolv (Beckman Instruments, Fullerton, Calif., U.S.A.).

Sucrose density gradient centrifugation

Samples were centrifuged in a linear sucrose gradient (5–20% sucrose) containing GuHCl as described by Kimata et al. (1974). Proteoglycans obtained by sequential extraction were used in this study. Fractions 1 & 2 were dialysed against water, lyophilized and again dissolved in the appropriate GuHCl buffer (fraction 1 in 0.4 M and fraction 2 in 4.0 M-GuHCl). Two kinds of gradients were set up; an associative (0.4 M-GuHCl-sucrose) and a dissociative (4.0 M-GuHCl-sucrose). Fraction 1 dissolved in 0.5 ml of 0.4 M-GuHCl was layered on the associative gradient, while fraction 2 dissolved in 0.5 ml of 4.0 M-GuHCl was layered on the dissociative gradient. Centrifugation was performed in a Beckman Model L2-65B ultracentrifuge with a swinging bucket rotor (SW 27·1) at 20 °C for 28 h at 25000 rev./min. Tube contents were collected in 0.5 ml fractions. An aliquot of 0.1 ml from each fraction was spotted on a 2·3 cm disc of Whatman 3MM filter paper and dried. The dried discs were placed into scintillation vials containing 10 ml of scintillation cocktail (as described above) and counted.

The relative amount of chondroitin 4- and 6-sulfate in radioactive sulfate labeled material was estimated by the enzymic method of Saito, Yamagata & Suzuki, (1968).
Molecular sieve chromatography of proteoglycans

Chromatography was performed using Controlled Pore Glass beads (CPG-10-2500; Electronucleonics, Fairfield, N.J., U.S.A.) according to the procedures developed by Lever & Goetink (1976) with some modifications (Lash & Vasan, 1978). The elution buffer, 0-5 M sodium chloride, contained 0-02 % sodium azide. Unlike the original method, in the modified procedure the column was eluted by gravity flow at 0-35 ml/min. The eluate was collected in 0-5 ml fractions. An aliquot from each fraction was used to determine the radioactivity as described above. Proteoglycans for this study were obtained by extracting the cartilage with 4·0 m-GuHCl.

Preparation of A1-D1 proteoglycan monomers

Proteoglycan monomers (A1-D1) were obtained by the method of Hascall & Heinegård (1974). Proteoglycans extracted with 4-0 M-GuHCl were first dialysed against 100 volumes of 0-5 M-GuHCl (containing the proteolytic inhibitors) for 24 h at 4 °C. Aggregate (A1) fractions were then prepared with associative cesium chloride density gradient centrifugation (initial density 1·65 g/ml; 40000 rev./min 15 °C; 48 h type 40 rotor fixed angle). A1 fractions were subsequently partitioned in a dissociative gradient (initial density 1·54 g/ml; 40000 rev./min; 15 °C; 48 h type rotor fixed angle) to separate monomeric proteoglycans (A1-D1). Proteoglycan monomers were subjected to extensive dialysis and lyophilized.

Interaction of proteoglycan monomers with hyaluronic acid

Radioactive sulfate labeled proteoglycan monomers (obtained as mentioned above) in 4-0 M-GuHCl were mixed with 1 mg of unlabeled proteoglycan monomers obtained from 13-day embryonic chick sterna. To this, hyaluronic acid (1 mg/ml in 4-0 m-GuHCl) was added. Cock comb hyaluronic acid was kindly provided by Dr. Balaz. The mixtures, as well as controls without hyaluronic acid, were set at room temperature for 1 h. They were then exhaustively dialysed against 0-5 M sodium chloride at 4 °C, solvent conditions which facilitates the interaction of proteoglycans with hyaluronic acid (Hardingham & Muir, 1972). The samples were chromatographed on CPG-10-2500 and the eluted fractions analyzed for radioactivity.

RESULTS

Sequential extraction of proteoglycans

Figure 1 shows that there is synthesis of small amounts of proteoglycan aggregate as early as stage 12 and 18. There is a continuing decrease in the amount of [35S]sulfate incorporated (per mg of tissue) into fraction 1 (uPGM = unassociated monomers) from stage 12 (84 %) to stage 18 (72 %) to stage 24 (62 %) to stage 35 (10 %). Likewise there is a corresponding increase in the amount of [35S]sulfate incorporated into fraction 2 (aggregates); stages 12, 18, 24, and 35

Sequential extraction of proteoglycans
Fig. 1. Distribution of \[^{35}S\]sulfate incorporation into sequentially extracted (0.4 M and 4.0 M-GuHCl) proteoglycans. Fraction 1 represents small unassociated (monomeric) proteoglycans extracted with 0.4 M-GuHCl, and fraction 2 represents large (aggregate) proteoglycans which are dissociated and extracted as monomers with 4.0 M-GuHCl. Each value is the average of three determinations. Data for stages 18, 24 and 35 have been presented previously in different form (Vasan & Lash, 1977b). The number of embryos used for each determination were: stage 12, 400; stage 18, 200; stage 24, 150; stage 35, 24.

respectively contain 16%, 28%, 38% and 90%. Thus the ratio of fraction 1 to fraction 2 decreases during chondrogenesis (5:25, 2:6, 1:6, and 0:1).

Sucrose density gradient centrifugation

In order to compare the molecular weight of intact proteoglycans extracted with various GuHCl concentrations, proteoglycans were submitted to centrifugation in a linear sucrose gradient. Fraction 1 from stage 24 (extracted with 0.4 M-GuHCl) has predominantly small molecular weight proteoglycans (uPGM) since it is banding at a low buoyant density. Whereas, fraction 2 from this stage (extracted with 4.0 M-GuHCl) has proteoglycans (PGM) which are slightly larger in size since they band at a higher buoyant density (solid lines, Fig. 2). Fraction 1 (extracted with 0.4 M-GuHCl) from stage 35 contains small quantities of large proteoglycans, but is composed primarily of small proteoglycans banding at low buoyant density (Fr 1/35, Fig. 2). Fraction 2 (extracted with 4.0 M-GuHCl) has a profile typical of a mature cartilage in that most of the proteoglycans are large (Fr 2/35, Fig. 2). From the distribution pattern of proteoglycans at different buoyant densities it is seen that fraction 2 (4.0 M-GuHCl extraction, which
Fig. 2. Sucrose density gradient profile of labeled proteoglycans sequentially extracted from stage-24 and stage-35 embryonic chick limbs. Fraction 1 was extracted with 0.4 M-GuHCl and centrifuged under associative (0.4 M-GuHCl) conditions. This fraction represents the unassociated, small monomers (uPGM). Fraction 2 was extracted with 4.0 M-GuHCl and centrifuged under dissociative (4.0 M-GuHCl) conditions. This fraction represents the small monomeric components of the large proteoglycan aggregate. There is an increase in the proportion of proteoglycan aggregates (as shown by the dissociated monomers) with age (e.g. Fr 2/35).

dissociates the larger proteoglycan aggregates) from stage 24 has much smaller proteoglycans compared to similar fraction from stage 35. Fraction 1 from stage 35 has some proteoglycans which are larger in size, banding at the same buoyant density as fraction 2. This may be due to the dissociation of larger aggregates, even at low ionic strength (0.4 M-GuHCl). Such dissociation at low ionic salt concentration has also been observed in sternal cartilage proteoglycans extracted with 0.4 M-GuHCl and eluted on a CPG-2500 column chromatography (Lash & Vasan, 1978).

Proteoglycans were also extracted with 4.0 M-GuHCl from stage-24 and stage-35 limb buds and a molecular weight comparison was made on a sucrose gradient centrifugation. Stage-24 limb bud has two distinct size proteoglycan molecules, about 80% of which are low buoyant density and the remaining of intermediate density (Fig. 3). Stage-35 proteoglycans are predominantly of larger size, which band at a higher buoyant density (Fig. 3). Proteoglycans from mature cartilage are of similar size (cf. Lash & Vasan, 1977).

Glycosaminoglycan analyses

Table 1 shows the relative amount of the [35S]sulfate-labeled glycosaminoglycans synthesized by limb buds at various stages. There is an increased
Limb-bud differentiation

Fig. 3. Sucrose density profile of labeled proteoglycans extracted from stage-24 and stage-35 embryonic chick limbs using 4.0 M-GuHCl and centrifuged under dissociative (4.0 M-GuHCl) conditions. The dissociated proteoglycans from stage-35 limbs (dashed line) are larger than those from younger stage-24 limbs (solid line). In Figs. 2 and 3, 120 embryos were used for stage 24, and 24 embryos were used for stage 35. These profiles are representative of three separate experiments.

Table 1. Relative amounts of the various [35S]sulfate-labeled glycosaminoglycans synthesized by limb buds (% ± S.D.)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Ch 6-s†</th>
<th>Ch 4-s‡</th>
<th>Resistant*</th>
<th>Ch 6/Ch 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>73 ± 3</td>
<td></td>
<td>16 ± 1</td>
<td>87/13</td>
</tr>
<tr>
<td>18</td>
<td>60 ± 4</td>
<td>11 ± 3</td>
<td>18 ± 3</td>
<td>73/27</td>
</tr>
<tr>
<td>24</td>
<td>33 ± 2</td>
<td>22 ± 2</td>
<td>3 ± 1</td>
<td>34/66</td>
</tr>
<tr>
<td>35</td>
<td>40 ± 3</td>
<td>64 ± 3</td>
<td>9 ± 2</td>
<td>44/56</td>
</tr>
</tbody>
</table>

* [35S]Sulfate-labeled GAG which is not degraded by chondroitinase ABC or AC.
† Relative amount of radioactivity associated with 4-sulfated disaccharide residues after chondroitinase ABC and AC treatment.
‡ Determined as the relative amount of radioactivity associated with 6-sulfated chondroitinase ABC and AC treatment.

synthesis of chondroitin 4-sulfate with a corresponding decrease in chondroitin 6-sulfate as development progresses. The chondroitinase-resistant material declines until stage 24, and after the onset of chondrogenic stage (i.e. stage 24) the resistant material shows a slight increase (possibly due to keratan sulfate).

Molecular sieve chromatography of proteoglycans

Molecular sieve chromatography of proteoglycans synthesized by limb buds corroborated the results obtained with sequential extraction and sucrose density
Fractions (0.7 ml)

Fig. 4. Elution profiles of proteoglycans separated on a CPG10-2500 column. Using this form of molecular sieve chromatography, a more heterogeneous molecular profile is displayed. Tissues were extracted under dissociative (4.0 m-GuHCl) conditions, and chromatography was done under conditions which facilitate re-aggregation (0.5 m-NaCl) in Figs. 4–6. Stage 18, dotted line: stage 24, dashed line; stage 35, solid line. There is a proportional increase in aggregates (peaks at $V_\text{ex}$, void volume), and a corresponding decrease in monomeric forms (peak to right of graph) that is related to stages of chondrogenic differentiation.

Gradient centrifugation. Stage-18 limb bud incorporated a small amount of radioactive sulfate into the proteoglycans (PGA) excluded from the column, and the included material (uPGM) was about 85% of the total (Fig. 4). Stage-24 limb bud incorporated more (35%) of radioactive sulfate into the large proteoglycan aggregate that is excluded from the column while the remaining material (65%) is in the included fraction (Fig 4). This trend of increased radioactive sulfate incorporated into large excluded proteoglycan aggregates continued to stage 35. At this stage about 80% of the radioactive sulfate was incorporated into the excluded proteoglycan aggregate (Fig. 4). This study reveals the increase in the proportion of large molecular weight proteoglycan aggregates as the limb bud differentiates, with the concurrent decrease in the proportion of small molecular weight unassociated proteoglycan monomers.

**Interaction of proteoglycan A1–D1 monomers with hyaluronic acid**

Proteoglycan monomers from stage 24 and 35 were used in these studies. Stage-18 limb bud was not used due to insufficient material. In a previous report (Vasan & Lash, 1977) we have shown that stage-24 limb bud is capable of synthesizing one link protein whereas stage 35 synthesizes two. This process of biochemical differentiation was attributed to the synthesis of two link proteins, leading to the aggregation of proteoglycans. In the current experiments, the synthesis of hyaluronic acid binding region (which is essential for aggregate
Limb-bud differentiation

Fig. 5. CPG10-2500 column chromatography of proteoglycan monomers (A1–D1 fractions, see Materials and Methods) from stage-24 limbs (solid line). Upon the addition of hyaluronic acid, there is no change in the profile, indicating that there is no significant interaction (dashed line). It should be noted that the A1–D1 monomers consist of three size groups.

Fig. 6. CPG10-2500 column chromatography of proteoglycan monomers (A1–D1 fractions) from stage-35 limbs (solid line). Upon the addition of hyaluronic acid, it is seen that there is significant interaction, and larger aggregates are formed (dashed line). It should be noted that the smallest monomers (fractions 90–100) are no longer present in the older limb-buds.

formation) was examined. Mixtures of A1–D1 monomers and A1–D1 monomers plus high molecular weight hyaluronic acid were prepared under dissociative conditions, dialysed under associative conditions and chromatographed on a CPG10-2500 column. The proportion of proteoglycan bound to hyaluronate was determined from the amount of labeled proteoglycan in the region of void
volume compared with control proteoglycan chromatographed in the absence of hyaluronate.

Figure 5 shows that at stage 24, there is insignificant binding of proteoglycan monomers and hyaluronic acid. Proteoglycan monomers from stage 35, however, show a high binding capacity (Fig. 6). This shows that the A1–D1 proteoglycan monomers at stage 24 have very few or no hyaluronic acid binding protein regions and stage-35 monomers have considerable binding capacity.

**DISCUSSION**

This report describes experiments in which proteoglycans were isolated directly from chick limbs at different stages of development. Proteoglycans of various size classes can be sequentially extracted from cartilage using first a low ionic salt solution and then a higher ionic salt solution (Sajdera & Hascall, 1969; Simůnek & Muir, 1972; Vasan & Lash, 1977a; Lash & Vasan, 1978). Extracting the cartilage with 0.4 M-GuHCl removes unassociated proteoglycans of smaller molecular weight (uPGM) and 4.0 M-GuHCl dissociates proteoglycan aggregates and extracts them as monomers (PGM) (Vasan & Lash, 1977b; Lash & Vasan, 1978).

Using this sequential extraction method, early embryonic limb-buds (stage 12, 18, and 24) were found to synthesize proteoglycans (uPGM) mostly of smaller molecular weight (84%, 72% and 62% respectively) and less proteoglycan aggregates (16%, 28% and 38% respectively). These results show that limb-buds even at the earliest stages of differentiation synthesize proteoglycan aggregates. Small quantities of a cartilage-related proteoglycan fraction had been reported in cultures prior to the onset of chondrogenesis (Pennypacker & Goetinck, 1976). The proteoglycans made by stage-35 chick limbs are very similar to those of mature cartilage (Vasan & Lash, 1977b). As cartilage matures, the size of the proteoglycan molecules become larger. In an earlier report we have shown that this increase in the aggregate formation in differentiated cartilage is related to the synthesis of two link proteins (Vasan & Lash, 1977b). The major proteoglycan molecules synthesized by young limb buds have several distinct differences from those synthesized by stage-35 limbs. They are smaller in molecular size, contain a higher ratio of chondroitin 6-sulfate to chondroitin 4-sulfate and do not interact with hyaluronic acid.

Sucrose density gradient centrifugation has been used to determine the molecular weight of proteoglycans (Kimata et al., 1974; Okayama et al., 1976; Kitamura & Yamagata, 1976; Lash & Vasan, 1977; Vasan & Lash, 1977a). Such studies show that chick limbs at stage 24 incorporate radioactive sulfate predominantly into smaller molecules whereas older limbs (stage 35) incorporate radioactive sulfate into proteoglycans which are larger in size. Low ionic salt concentration of GuHCl (0.4 M) dissociates some of the proteoglycan aggregates. Such unexplained dissociation has been observed previously (Lash & Vasan,
Limb-bud differentiation

Kitamura & Yamagata (1976) have recently reported the isolation of a proteoglycan (PCS-M) which is similar to the one described in this paper (stage 24), banding at a higher buoyant density. Such a proteoglycan molecule from day-2 chick limb culture has also been described by Okayama et al. (1976). Kitamura & Yamagata (1976) have reported that they were unable to detect any small molecular weight proteoglycans (PCS-L) before the initiation of chondrogenesis. Contradictory to this, the present report as well as that of Palmoski & Goetinck (1972) and Goetinck et al. (1974) demonstrate the presence of small molecular weight proteoglycans in embryonic limbs (this paper) and somites (Lash & Vasan, 1977, 1978).

Molecular sieve chromatography using Sepharose (Hardingham & Muir, 1972; Hascall & Heinegård, 1974; Lash & Vasan, 1977), Biogel (Palmoski & Goetinck, 1972; Goetinck et al. 1974) and controlled pore glass beads (Lever & Goetinck, 1976; Lash & Vasan, 1977; Vasan & Lash, 1977a) has been successfully adopted to determine the molecular weight of proteoglycans and to isolate different molecular sizes. In this paper, CPG10–2500 was used to characterize proteoglycans. Chick embryo limb buds at stage 18 incorporate 18% of radioactive sulfate into large proteoglycans which are characteristically seen in fully differentiated cartilage. Stage-24 limb buds incorporate radioactive sulfate into proteoglycan aggregates to the extent of 35%. Such a trend of increased radioactive sulfate incorporation into proteoglycan aggregates and decreased incorporation into small proteoglycan monomers appears to be a differentiation phenomenon.

It is not clear why the proteoglycans in the excluded volume of stage-24 extracts, which chromatograph in the region of aggregates (Fig. 4, dashed line), behave as non-aggregating molecules when prepared as A1–D1 monomers. The intermediate size proteoglycan monomers (Fig. 5) are undoubtedly derived from the A1 (‘aggregate’) fraction. The fact that these monomers do not bind significantly with the addition of hyaluronic acid indicates that, although they chromatographed in the excluded volume, they were not conventional aggregates. The methodology of proteoglycan isolation is such that it is not yet known why a small proportion of non-aggregated proteoglycans pass through a molecular sieve column in the excluded volume (V. Hascall, personal communication). If these proteoglycans were truly aggregated, it would indicate that the HA-binding region was quite labile in the early stages and more stable in differentiated cartilage. Regardless of the interpretation, it is unequivocal that the A1–D1 monomers from stage-24 limb buds are predominantly lacking in the HA-binding region, whereas at stage 35 most of the A1–D1 monomers contain a stable HA-binding region of the core protein.

In an earlier paper (Vasan & Lash, 1977b) the appearance of aggregates was attributed to the synthesis of two link proteins which causes the aggregation of proteoglycan monomers, and not due to the synthesis of new species of cartilage specific proteoglycans. Whereas one link protein was detected prior to
stage 24, two link proteins were detected after stage 24. Proteoglycans synthesized by embryonic chick chondrocytes in culture are heterogeneous, and one class of proteoglycans may be affected by inhibitors of chondrogenic expression. This suggests the possibility that the affected proteoglycans may represent a ‘cartilage specific’ species (Palmoski & Goetinck, 1972; Levitt & Dorfman, 1973; Okayama et al. 1976). Analysis of chick limb buds in vivo as reported in this paper, and embryonic limb-bud cells (Goetinck et al. 1974) in which chondrogenic expression takes place, suggests that the cartilage specific event might not be the appearance of a new species of proteoglycan, but rather the preferential increase of a pre-existing species, as well as the synthesis of two link proteins (Vasan & Lash, 1977) and the synthesis of a stable hyaluronic acid binding region.

Thus the lack of predominant quantities of proteoglycan aggregates in the younger limb buds (stages 12, 18, 24) can be related to one or both of the following: Presence of only one of the two link proteins and/or the lack of hyaluronic acid binding region of the core protein of the proteoglycan monomers. The results of proteoglycan monomer interaction with hyaluronic acid confirm the fact that embryonic limb buds (stage 24) synthesize proteoglycans which are not capable of interacting with hyaluronic acid when prepared as A1–D1 monomers. This may be due to the lack of, or instability of, the hyaluronic acid binding region. Stage-35 limb-bud proteoglycans show a maximum binding with hyaluronic acid. It can be inferred from this that the gene for hyaluronic acid binding protein synthesis is being expressed only in differentiated chondrocytes. This is also in agreement with the report that the core protein is a heterogeneous molecule (Hardingham et al. 1976). Finally it leaves us with the view that the proteoglycan monomers synthesized by young limb buds are indistinguishable from those seen in the differentiated limb chondrocytes. Studies are in progress to further understand the regulatory events during embryonic limb chondrogenesis.

Supported by NIH research grant HD-00380 to J.W.L.

REFERENCES


(Received 16 June 1978, revised 20 September 1978)