

Electron Microscopic Characterization of Chick Embryonic Skeletal Muscle Proteoglycans

DAVID G. PECHAK, DAVID A. CARRINO, and ARNOLD I. CAPLAN
Department of Biology, Case Western Reserve University, Cleveland, Ohio 44106

ABSTRACT In this article, proteoglycans from embryonic chick leg muscle are quantitatively and qualitatively compared with day 8 high density cell culture cartilage proteoglycans by electron microscopy of proteoglycan–cytochrome *c* monolayers. The visualized proteoglycan profiles were separated into four categories according to shape, size, and complexity. The two major categories were further characterized by lengths of core proteins, lengths of side projections, and distance between side projections. Two large proteoglycans are identifiable in spread leg muscle preparations. One group has a core protein (mean length of 205 nm) from which extend long thin side projections that we interpret to be groups of chondroitin sulfate glycosaminoglycans with a mean length of 79 nm. This large chondroitin sulfate proteoglycan is the only type found in muscle cultures as determined both biochemically in the past and now by electron microscopy and is referred to as muscle proteoglycan. The second large proteoglycan has a mean core protein length of 250 nm and side projections that are visibly shorter (mean length of 38 nm) and thicker than those of the muscle proteoglycan. This group is referred to as the mesenchymal proteoglycan since its biosynthetic origin is still uncertain. We compare these two profiles with the chick cartilage chondroitin sulfate proteoglycan that has a mean core protein length of 202 nm and side projections with a mean length of 50 nm. The data presented here substantiate the earlier biochemical characterization of these noncartilage proteoglycans and establish the unique structural features of the muscle proteoglycan as compared with the similar profiles of the cartilage and mesenchymal proteoglycans.

The Kleinschmidt cytochrome *c* monolayer spreading technique (20, 32, 33) has been used to visualize and characterize proteoglycans. These studies have concentrated on cartilage (2, 4–6, 25, 28, 32–34) or cartilage tumor proteoglycans (3, 17) and have reported on the size and complexity of aggregates (3, 4, 6, 17, 32, 33), changes in proteoglycans with age or anatomical location (2, 5, 25, 28), and differences between organisms (4). In this context, this laboratory has reported details of transmission electron microscope studies and biochemical characterization of day 8 high density chick limb bud culture chondrocyte proteoglycans (28). The day 8 chick limb bud chondrocyte proteoglycan has a molecular weight of $\sim 2 \times 10^6$ with chondroitin sulfate chains of $\sim 20,000$ mol wt, which contain 67% 6-sulfated chondroitin sulfate. Although cartilage and chondrosarcoma proteoglycans have received substantial attention, proteoglycans from several tissue sources have been characterized biochemically (1, 11, 12, 14, 15, 18, 19, 22, 23, 27, 29, 31, 35–37).

We recently biochemically characterized newly synthesized chick cartilage and muscle proteoglycans isolated from embryos (12, 14) as a means of comparing their structure with that of the proteoglycans synthesized in cell cultures (11). In the study of *in vivo* proteoglycans synthesized in leg muscle, three distinct classes of proteoglycans were identified (11, 12). By Sepharose CL-2B chromatography of D1 fractions, both large ($K_{av} = 0.14$ – 0.23) and small ($K_{av} = 0.77$ – 0.81) proteoglycans were found. The large proteoglycans could be further resolved upon analysis of the glycosaminoglycans by Sepharose CL-6B chromatography which revealed the presence of two classes of chondroitin sulfate whose parent molecules were referred to as muscle proteoglycan *A* and muscle proteoglycan *B* (12). From the data presented here and elsewhere, we now can state that the *A*-type proteoglycan is synthesized by skeletal muscle cells, and it is hereafter referred to simply as muscle proteoglycan. The origin of the *B*-type proteoglycan is still not known and we hereafter refer to it as mesenchymal

proteoglycan to signify that it is synthesized by cells of mesenchymal origin. Muscle cells, fibroblasts, satellite cells, or other connective tissue cells are, therefore, not excluded. These assignments can be made because the three classes of proteoglycans have distinctive spreading characteristics and morphologies as viewed in the electron microscope and reported here.

The mesenchymal proteoglycan is similar to that of cartilage proteoglycan in a number of biochemically determined structural features, whereas the muscle proteoglycan differs significantly in several parameters (Table I). The average hydrodynamic size of the monomers, determined by Sepharose CL-2B chromatography, is much larger for the muscle proteoglycan relative to cartilage and mesenchymal proteoglycans, which are similar to each other in monomer size. The chondroitin sulfate chains are also similar in size for the cartilage and mesenchymal proteoglycans and substantially smaller than those of the muscle proteoglycan. It is important that a major difference is found in the 6-sulfated chondroitin sulfate, which is unusually high for the muscle proteoglycan, 90% as compared with 66 and 60% for the cartilage and mesenchymal proteoglycans, respectively.

To characterize more fully the proteoglycans synthesized in chick muscle tissue *in situ* and substantiate the biochemical studies, the visual technique of proteoglycan-cytochrome *c* monolayer spreading and transmission electron microscopy has been employed. The usefulness of proteoglycan-cytochrome *c* spreading of macromolecules has been amply demonstrated with cartilage proteoglycans in the work of Rosenberg et al. (32, 33) and Buckwalter et al. (3-6) among others. One major advantage of this technique is the ability to evaluate individual molecules, both aggregates and monomers. Although numerous individual profiles must be examined to determine average parameters for a large population, the technique allows quantitative comparison of populations from different developmental stages (5), different tissues (2), or, as reported here, subpopulations within one tissue.

The study reported here involves both a qualitative and quantitative aspect, because at least three proteoglycan types have been described biochemically for *in ovo* muscle tissue (12). Since the technique of proteoglycan-cytochrome *c* spreading involves the evaluation of molecules on an individual basis, it is relatively easy to separate the biochemically characterized types by their morphological appearance in monolayer spreads. Moreover, the technique of proteoglycan-cytochrome *c* spreading, which has been successfully and valuably applied to the examination of cartilage proteoglycans (2, 5, 25, 35), is extended into the study of noncartilage proteoglycans, an area in which only a modest application has been made (26, 36).

MATERIALS AND METHODS

Isolation of Proteoglycans: The details of isolation and purification procedures for the proteoglycans used in this study have been presented elsewhere (11, 12, 16, 21). Muscle tissue proteoglycans were isolated either from culture material or from whole embryos. The cultures consisted of day 11 chick myoblasts prepared as done previously (7) and maintained until after fusion of myoblasts into myotubes, day 3. The leg muscle was dissected from embryos of the desired age. The source of cartilage proteoglycans was day 8 high density cultures of chick limb bud mesenchymal cells in which most of the cells are chondrocytes that synthesize proteoglycans whose structure closely resembles those of animal hyaline cartilage (9, 16, 21, 28). In all cases, the proteoglycans were extracted with 4 M guanidinium chloride containing protease inhibitors. The density of the clarified extracts was adjusted with CsCl to

TABLE I
Biochemical Features of the Proteoglycans from Cartilage and Muscle

| | Cartilage | Muscle | Mesenchymal |
|------------------------------------|-----------------|-----------------|-------------------|
| Monomer | | | |
| K_{av} , Sepharose CL-2B | 0.25 | 0.1 | 0.2 |
| Estimated molecular weight | 2×10^6 | 4×10^6 | 2.5×10^6 |
| Chondroitin sulfate | | | |
| K_{av} , Sepharose CL-6B | 0.50 | 0.26 | 0.47 |
| Estimated molecular weight | 20,000 | 70,000 | 24,000 |
| CS chains/monomer | 80-100 | 40-60 | 80 |
| 6-Isomer/total chondroitin sulfate | 66% | 90% | 60% |
| Keratan sulfate | | | |
| K_{av} , P-30 | 0.19 | | |
| Estimated molecular weight | 7,000 | | |
| KS chains/monomer | 20-30 | 0 | 0 |
| Oligosaccharides | | | |
| O-linked/monomer | 90-120 | 600* | ND |
| N-linked/monomer | 25-35 | 40* | ND |
| Aggregability with hyaluronate | + | + | ND |

Data were taken from References 8, 9, 11, 12, 14, 16, and 21 and from work in progress. Cartilage proteoglycan data are for the molecules synthesized by chondrocytes in day 8 high density cultures of stage 24 chick limb mesenchymal cells. Skeletal muscle proteoglycan data are for the molecules synthesized by myotubes in day 3 leg muscle cultures, although those parameters that have been examined with *in ovo* leg muscle proteoglycans are the same as those of the *in vitro* molecules. Data for mesenchymal proteoglycan are from *in ovo* leg muscle. ND, not determined.

* 15 O-linked and 1 N-linked oligosaccharide per chondroitin sulfate chain.

1.50 g/ml, and the extracts were centrifuged to equilibrium in a Beckman SW 50.1 or 50 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) at 35,000 rpm and 10°C for 44-46 h. The gradients were fractionated into quarters with a Beckman tube slicer and the fractions were dialyzed and lyophilized. For the studies reported here, we analyzed only the high density proteoglycans recovered in the D1 fraction (24) which had a density of ≥ 1.60 g/ml.

Morphological Examination of Proteoglycans: Lyophilized samples of purified proteoglycans were dissolved and stored overnight at 4°C in 0.5 M sodium acetate, pH 7, at a concentration of ~ 100 μ g/ml. The proteoglycan solution was diluted 1:10 with 1 M ammonium acetate of which 75 μ l was mixed with 20 μ l of a solution containing cytochrome *c* at a concentration of 0.5 mg/ml in 0.01 M Tris(hydroxymethyl)aminomethane, 0.001 M ethylenediaminetetraacetic acid, pH 8.5. The resulting solution was placed on a wet glass slide and allowed to spread over a hypophase of 0.3 M ammonium acetate, pH 5. The resulting monolayer was picked upon on freshly carbon-coated nitrocellulose films and stained in 0.2% (wt/vol) uranyl acetate in 90% ethanol for 1-2 min then rinsed in 90% ethanol. Some grids were also rotary shadowed with platinum-palladium which was wrapped around a pointed tungsten filament and placed 8 cm from and 10° above the rotating grids.

Grids were examined and micrographs were taken with a JEOL 100S transmission electron microscope operating at 60 kV. Nonoverlapping fields were recorded on film at a magnification of 26,300 and photographically enlarged to $\times 72,000$ for tracing onto clear acetate. All profiles that could be accurately traced were included. Acetate sheet tracings were machine copied to white paper and individually cut out. The individual profiles of proteoglycans were categorized according to size, shape, and complexity to derive the collage figures.

For quantitative analysis, electron microscope images were photographically enlarged by 128,000. Magnifications were calibrated by means of a diffraction grating. Proteoglycans corresponding to specific categories (III and IV of muscle and III of cartilage) were traced with an acoustical digitizing pen. Measurements recorded included length of core protein, length of side projections (corresponding to groups of glycosaminoglycans), and distance between side projections. Statistical analysis was performed on a PDP-11/34 computer (Digital Equipment Corp., Maynard, MA).

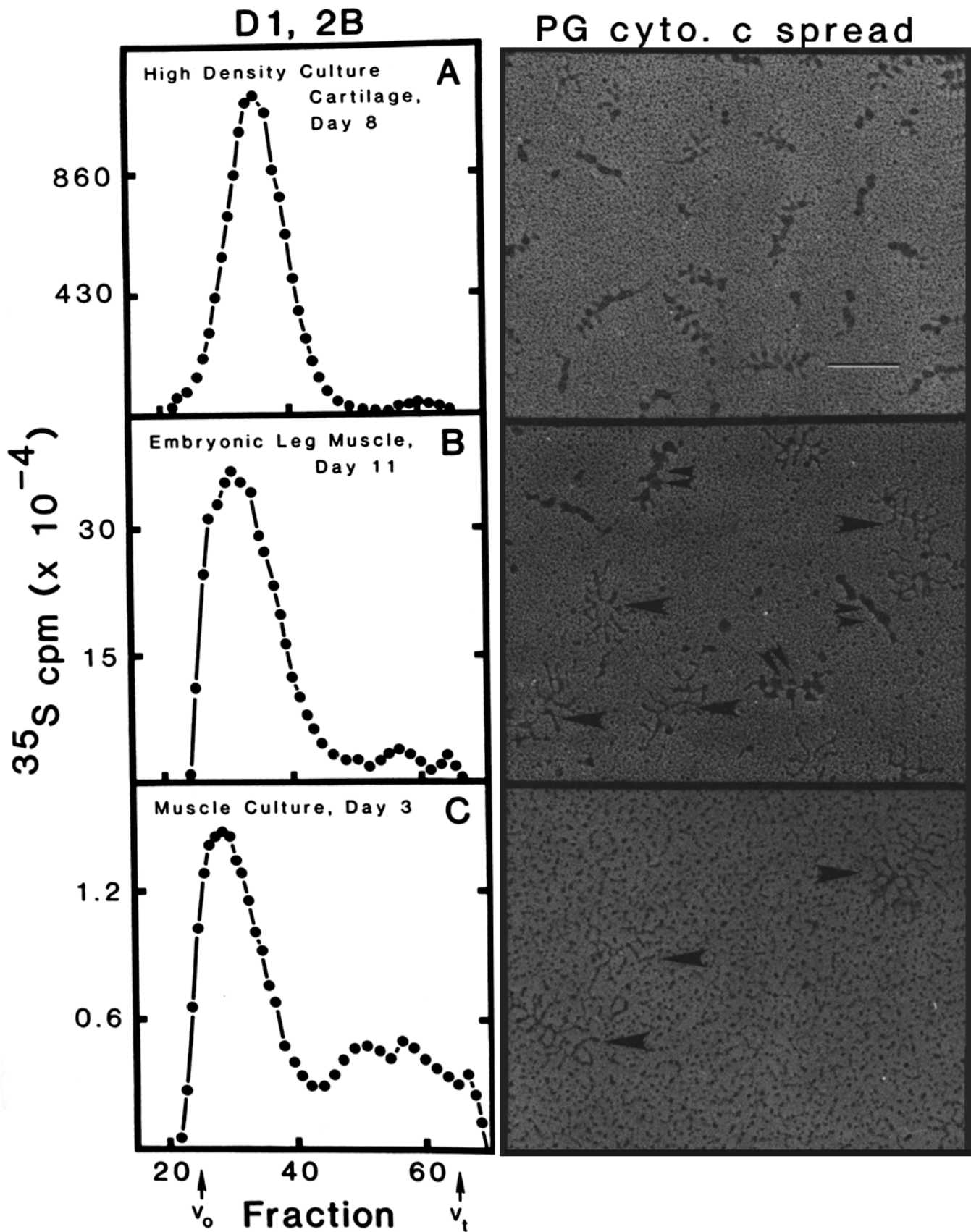


FIGURE 1 Sepharose CL-2B profiles with corresponding cytochrome c (cyto. c) spread molecules. (A) Day 8 high density culture chondrocyte proteoglycans. The major molecule in the spread preparation is a larger proteoglycan with short side projections. (B) Day 11 embryonic leg muscle proteoglycans (PG). The two major proteoglycans are both relatively large, but one has long, thin side projections (arrow), and the second has short, thick side projections or swellings (double arrowheads). (C) Day 3 myogenic culture proteoglycans. Only one type of large proteoglycan (arrowheads) is present; it has long thin side projections. Note that the column profiles represent only newly synthesized molecules and comparable results have been reported previously (11, 12, 21). Bar, 250 nm. V_0 , void volume; V_t , total volume.

RESULTS

Qualitative Characterization

Fig. 1 shows the Sepharose CL-2B elution profiles and corresponding transmission electron micrographs of cytochrome *c* spread monolayers of the three proteoglycan samples examined in this article. These three proteoglycans are all chondroitin sulfate proteoglycans. Day 8 high density culture cartilage proteoglycans (Fig. 1A) have been detailed elsewhere (21, 28) and represent a standard with which other chick proteoglycans can be compared. The major peak has a K_{av} of 0.25 and the electron micrograph presents a random population of largely uniform images whose side projections are short or collapsed and appear as bumps on both sides of

the long axis (the core protein). Leg muscle proteoglycans from day 11 embryos (Fig. 1B) show a different Sepharose CL-2B profile (11, 12, 14) and present two clearly identifiable images of large proteoglycans. One image is similar to those observed with cartilage proteoglycans with short or collapsed side projections (double arrowheads), and the other has noticeably extended side projections that are perceptibly thinner and arranged along the axis or core protein (single arrowheads).

In spreads of molecules isolated and purified from muscle cultures, only the images with long, thin, well-spread side projections are observed (Fig. 1C, arrows). In no case is the other type of large proteoglycan observed in the muscle culture preparations. Biochemical analysis of the proteoglycans

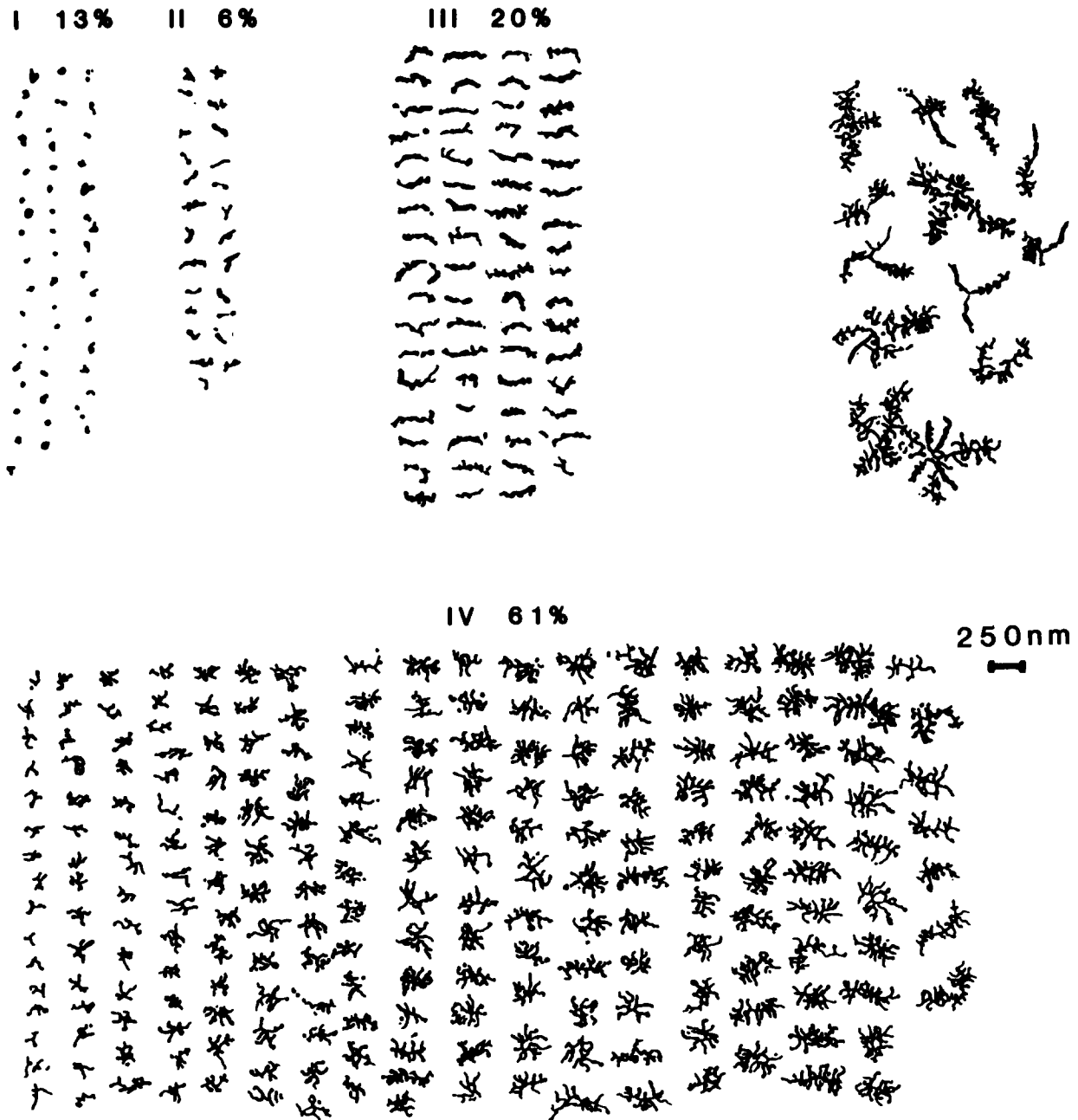


FIGURE 2 Collage of 386 molecular profiles of day 11 leg muscle proteoglycans from the D1 fraction of a cesium chloride density gradient. Tracings were made from cytochrome *c* spread preparations and categorized according to size, shape, and complexity. Criteria for the various categories are explained in the text. Bar, 250 nm.

synthesized by the cells in muscle cultures reveals that the large chondroitin sulfate muscle proteoglycan is produced with the complete absence of synthesis of the mesenchymal proteoglycan (11, 12, 14). Based on these qualitative observations and the quantitative measurements provided below, we are confident in assigning the cells of origin of the two large proteoglycan images seen in the spreads of *in vivo* leg muscle molecules. Because of the structural similarities between cartilage proteoglycan and mesenchymal proteoglycan (the chondroitin sulfate chains of similar size and sulfation pattern; see Table I), we feel that the more condensed images seen in the spreads of day 11 embryo leg muscle material, which resemble the major images observed in cartilage proteoglycan spreads, represent molecules of mesenchymal proteoglycan. The fact that only one morphology, that of the highly spread molecules, is seen in preparations from muscle cultures argues against the possibility of artifacts that result in "collapsed" and "expanded" images in cytochrome *c* spreads of *in vivo* day 11 leg muscle proteoglycans and substantiates our identification of the highly spread images as muscle proteoglycans.

Proteoglycans isolated from leg muscle of day 11 chick embryos (D1 molecules) were prepared on carbon films and morphologically categorized as outlined in Materials and Methods. A total of 386 monomer profiles were recorded and placed into one of four categories. Although any given profile may be placed into one of several areas of the collage, the arrangement in Fig. 2 is a fair representation of the major types and proportions of proteoglycans present within the sample. The arrangement is designed to give order to the random appearance of proteoglycan molecules as seen by electron microscopy. We evaluated all recognizable proteoglycan profiles so as not to bias the results toward a particular type of profile. Self-aggregated profiles, although grouped together in the collage (upper right portion of Fig. 2), were placed in their respective categories as monomers to derive the stated percentages. This ordering allows a quantitative

view of the distribution of all macromolecules present in the sample.

Category I, which represents 13% of the total, can be described as comprising relatively small profiles that range from 20 to 110 nm with no apparent axis (Fig. 2). Category II also comprises relatively small units (from 60 to 150 nm) but an axis is discernible, although side projections are absent or ill-defined; this group comprises 6% of the total. Category III, which represents 20% of the sample, is characterized by profiles with a long axis-to-diameter ratio of at least 3; the long axes range from 150 to 300 nm and side projections, if present, are short or, more often, visualized as swellings or bumps along the long axis. The average length and standard deviation of these profiles as measured from the collage are 247 and 48 nm, respectively. Category IV, comprising 61% of the total, has been put in a profile gradient from relatively simple profiles that are short with few side projections to complex profiles with relatively long axes and numerous side arms. As a whole, this group has long, well-extended side projections and the molecules, as measured from the collage, range in length from 89 to 658 nm, while the mean length is 282 nm with a standard deviation of 104 nm.

The collage reveals that the variability in the length of molecules in category III is small as compared with that of category IV. Micrographs illustrating the differences seen in the degree of spreading within the major categories, III and IV, exemplify this variability (Fig. 3). Category III is represented by a molecule 250 nm long with collapsed or short side projections (Fig. 2, A-C), and category IV is characterized by molecules that vary greatly in length but, regardless of length, exhibit exceptionally long, thin side projections (Fig. 3, D-F). These category IV molecules are relatively insensitive to the properties of the supporting substrate, in that even with little or no carbon present on the nitrocellulose films, the molecules with long, thin side projections can be clearly visualized (data not shown), whereas category III molecules exhibit spread side projections only under optimum condi-

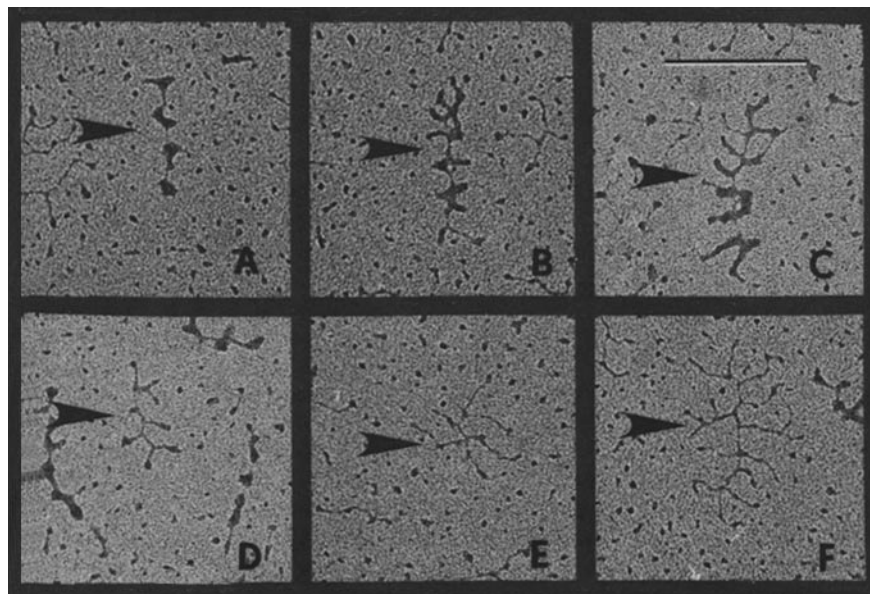


FIGURE 3 Cytochrome *c* spread molecules of day 11 leg muscle proteoglycan monomers from the D1 fraction of a cesium chloride density gradient. In A-C are molecules that illustrate the various degrees of side-projection spreading of the mesenchymal proteoglycan (category III molecules of Fig. 2). In D-F are molecules that represent muscle proteoglycan (category IV of Fig. 2). Bar, 250 nm. $\times 73,600$.

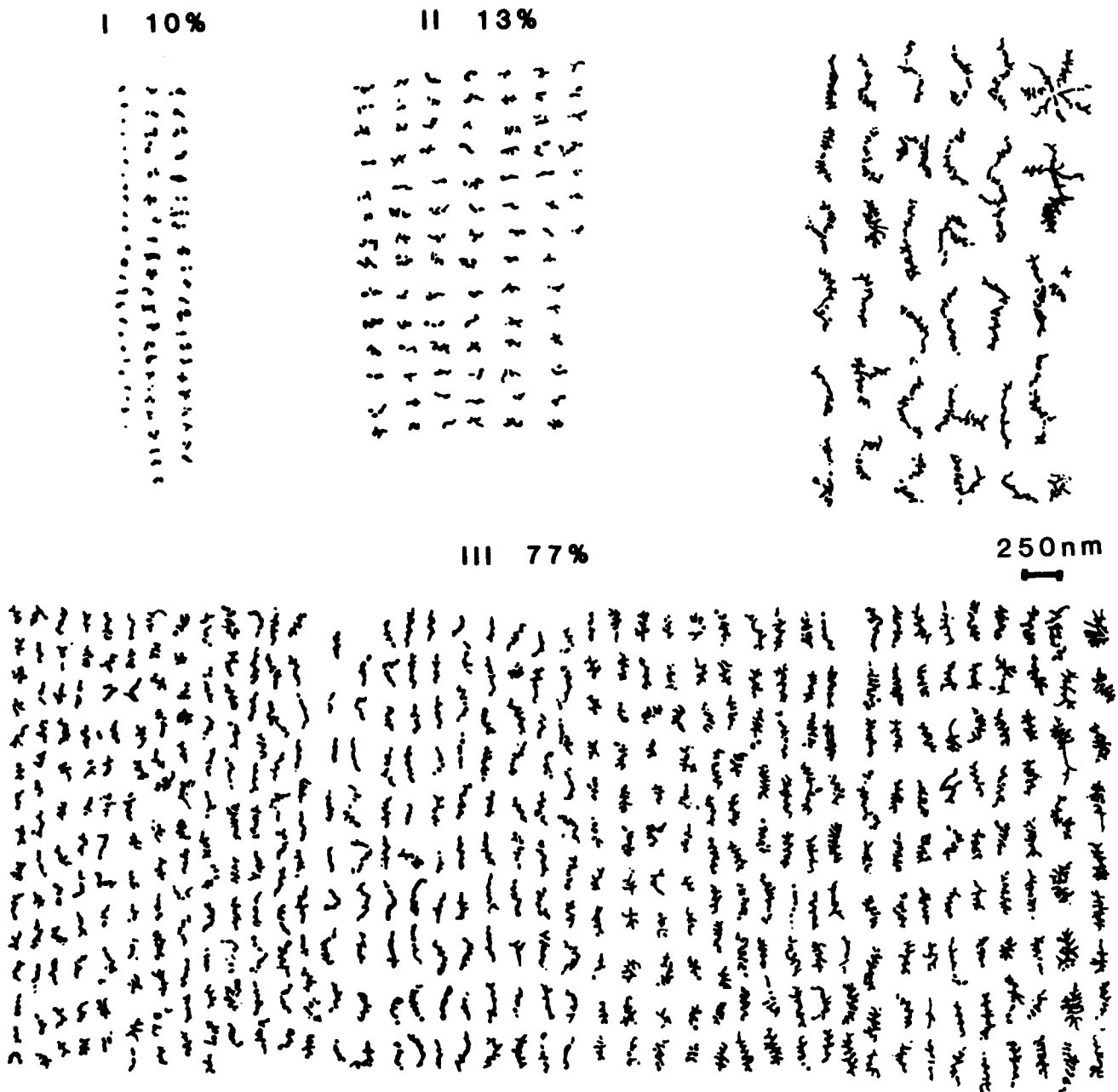


FIGURE 4 Collage of 634 molecular profiles of day 8 high density culture chondrocyte proteoglycans from the D1 fraction of a cesium chloride density gradient. Tracings were made from a cytochrome c preparation and categorized according to size, shape, and complexity. Bar, 250 nm.

tions and, at all times, the side projections are thicker and shorter than those of the category IV molecules.

Although the above categories may be artificial groupings of molecules, they permit further analyses of spread molecules as groups rather than a heterogeneous and random mixture of images. Categories III and IV, which represent ~80% of the profiles present, were further analyzed. Although the actual decision to relate these two categories to groups known by biochemical characteristics was not made until all morphological analyses were completed, category III molecules will be described as mesenchymal proteoglycans, category IV molecules as muscle proteoglycans. These morphological types correspond to the classification of the two major proteoglycans biochemically described in developing muscle (12).

Cartilage Proteoglycans as a Standard

Detailed biochemical and electron microscopic studies of chick limb bud chondrocyte culture proteoglycans have been published (16, 21, 28). For comparison and because we consider these molecules to be standards, we undertook an electron-microscopic analysis similar to that described above for the muscle proteoglycan. A total of 634 profiles were recorded which could be grouped into three categories (Fig. 4). Monomers of self-aggregated profiles (upper right portion of Fig. 4) were separately traced and placed in their respective categories as monomers, which increased the number of categorized monomers to 683. Categories I and II correspond to the comparable groups for leg muscle (Fig. 2). The lengths range

from 11 to 118 nm for category I, which represents 10% of the total population, and from 58 to 190 nm for category II, which represents 13% of the total. Category III, which represents 77% of this sample, is characterized by a long axis-to-diameter ratio of at least 3. The long axes range in size from 73 to 391 nm and side projections are visualized as a gradient from mere swellings along the long axis to extended side projections. The average length and standard deviation of these profiles as measured from the collage are 206 and 57 nm, respectively. Category III can be characterized as a gradient from relatively simple profiles that are short with few side projections to complex profiles of long axis and numerous side projections.

Examples at higher magnification from this cartilage D1 preparation representative of molecules grouped in category III are shown in Fig. 5. We believe that all of the profiles of category III represent the same proteoglycan type, but due to variability in the degree of spreading of the side projections (assumed to be groups of chondroitin sulfate glycosaminoglycans), a gradient exists from dense unextended molecules (Fig. 5A) through less dense profiles with maximally extended side projections (Fig. 5D), although the latter are, nevertheless, clearly distinct from the leg muscle proteoglycan category IV images described above (compare Fig. 5D with 3F.) The quantitative data presented below were obtained from such micrograph profiles rather than collage profiles. Due to difficulty in accurately measuring side projection lengths of many of the category III profiles, all data were collected from the most spread and clearly defined profiles as represented in Fig. 5D.

Quantitative Characterization

Profiles of day 11 leg muscle molecules representing mesenchymal and muscle proteoglycans (categories III and IV, respectively) were selected for detailed analysis. Micrographs were enlarged to give final magnifications of 100,000–125,000 and the molecular profiles were traced with a digitizing pen as described in Materials and Methods. Since mesenchymal proteoglycan molecules exhibit extended side projections only rarely, molecular profiles were selected that had such side projections so that they could be measured with reasonable accuracy. Therefore, the resulting data represent the maximally extended side projections of this group (Fig. 3, B and C), whereas measurements of axis length were determined separately for molecules without extended side projections (Fig. 3A) as well as for molecules with extended side arms.

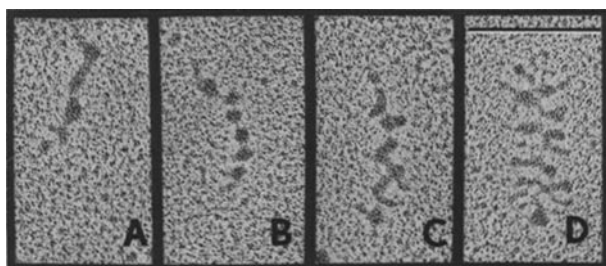


FIGURE 5 Cytochrome c spread molecules of day 8 high density culture chondrocyte proteoglycan molecules. A–D represent molecules of category III (Fig. 4) and illustrate the various degrees of side-projection extension found in this group. All histogram data (Figs. 6–8) were collected from molecules of the type seen in D. Bar, 250 nm. $\times 70,000$.

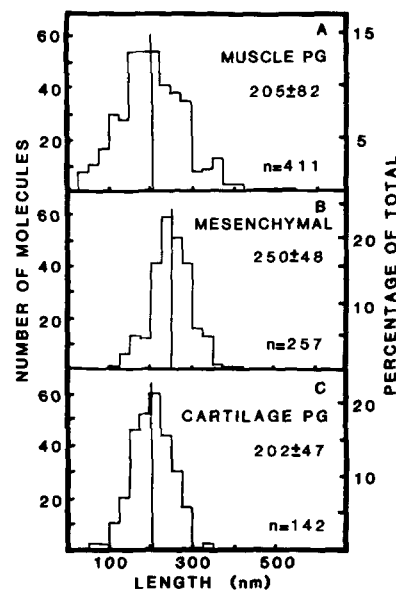


FIGURE 6 Histograms of core protein length. Vertical bars indicate mean value. (A) Day 11 leg muscle, muscle proteoglycan. (B) Day 11 leg muscle, mesenchymal proteoglycan. (C) Day 8 high density cell culture, cartilage proteoglycan.

The resulting mean and standard deviations for these length measurements are the same (250 ± 48 ; 258 ± 44 nm). In a comparison of molecules of different types, we selected the category III molecules with more extended side projections for analysis of the lengths of the axes (assumed to be equivalent to core protein length), lengths of the side projections (assumed to be groups of glycosaminoglycan chains), and the distance between the side projections. In this analysis of the muscle proteoglycan molecules (category IV), the difficulty was in obtaining accurate determinations of the core protein component of some molecular profiles, since the diameter of the side projections was often the same as that of the core protein. Data for the muscle proteoglycan were obtained from molecular profiles in which the core protein could be clearly discriminated from the side projections, regardless of the core protein length.

The results of such analysis are shown as histograms (Figs. 6–8). The axis (core protein) of the muscle proteoglycan monomers, measured from 411 images, varies widely (Fig. 6A). The mean length was determined to be 205 nm with a standard deviation of 82. The minimum and maximum axis lengths were 25 and 525 nm, respectively. Lengths of 257 molecules of the mesenchymal proteoglycan were likewise determined (Fig. 6B). Although the lengths range from a low of ~ 100 nm to a high of 425 nm, 80% were between 200 and 300 nm, with an average length of 250 nm and a standard deviation of 48. The shapes of the two histograms reflect the contrast in variability of the core protein lengths in the category III and category IV molecules. (Compare Fig. 6, A with B.) For comparative purposes, the core protein lengths of day 8 high density chick limb bud culture cartilage proteoglycan has a mean value of 202 nm (Fig. 6C), very close to that of the muscle proteoglycan. However, the shapes of the two histograms differ in that the cartilage culture material has a much smaller variability with a standard deviation of 47.

The lengths of core proteins of chick cartilage proteoglycan have been reported by Kimura et al. (28). Their measurements made from aggregated and reaggregated monomers are con-

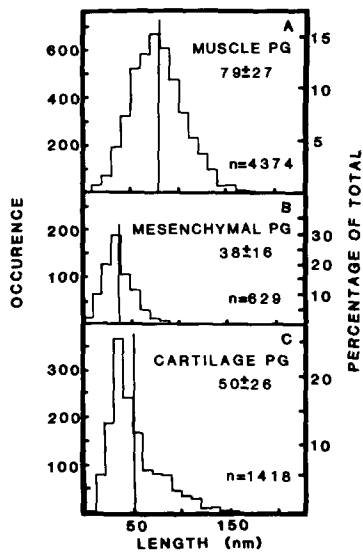


FIGURE 7 Histograms of side projection lengths. Vertical bars indicate mean value. (A) Day 11 leg muscle, muscle proteoglycan. (B) Day 11 leg muscle, mesenchymal proteoglycan. (C) Day 8 high density cell culture, cartilage proteoglycan.

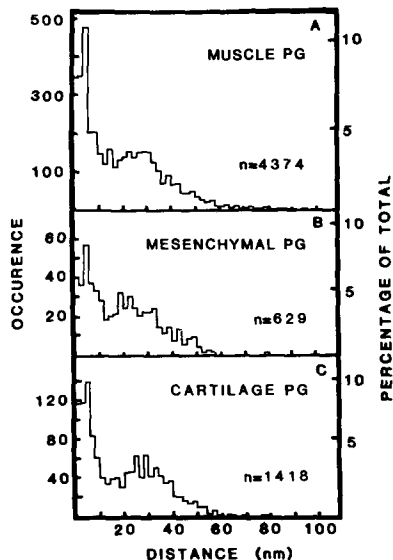


FIGURE 8 Histograms of distance between side projections. (A) Day 11 leg muscle, muscle proteoglycan. (B) Day 11 leg muscle, mesenchymal proteoglycan. (C) Day 8 high density cartilage culture, cartilage proteoglycan. See text for description.

sistently longer than those reported here. A partial explanation could be that proteoglycans in aggregate form often exhibit a hyaluronic acid binding region that attaches the chondroitin sulfate-rich region to hyaluronic acid (4). When monomeric proteoglycans are identically spread for electron microscopy, the hyaluronic acid binding region is less clearly visible or has folded back on itself, making the molecule appear shorter. To determine if the aggregated versus dissociated conditions of proteoglycans could explain the differences in size, we measured the core protein lengths of day 8 culture cartilage proteoglycan aggregates by the means used in this study. The lengths of proteoglycan monomers measured in such aggregates were only slightly longer than those measured as dissociated monomers (225 ± 69 vs. 202 ± 48 ; data not shown). We can offer no obvious explanation for the discrepancy in

the core protein lengths measured in this analysis and the previous one. Inclusion of catalase crystals on the carbon film of the grids used for spreading would provide a more accurate determination of the magnification, but such a method was not employed in the previous study of these proteoglycans in which the magnification was calibrated in a manner similar to that used here (28).

The muscle proteoglycan has a mean side projection length of 79 nm with a standard deviation of 27, and the shape of the histogram is symmetrical around the mean (Fig. 7A). The mean side projection length for the mesenchymal proteoglycan is 38 nm with a standard deviation of 16, and the shape of the histogram is somewhat skewed to the lower values (Fig. 7B). Cartilage proteoglycans have a mean side projection length of 50 nm with a standard deviation of 26, and the shape of the histogram is skewed heavily toward the lower values. The nonsymmetrical shape of the histograms around the means is an indication of the number of partially collapsed, or not fully expanded, side projections in the latter two groups. The biochemical characteristics of the glycosaminoglycans in the mesenchymal and cartilage proteoglycan may be responsible for the shorter and thicker appearance of the side projections when spread by the cytochrome *c* technique.

Fig. 8 illustrates a similarity of the three proteoglycans under study. The distance between side projections was determined irrespective of the side of the core protein from which projections extended. Therefore, two adjacent projections extending from opposite sides of the axis represent a distance between side projections of zero. Since the frequency of this situation is large, most values are between 0 and 10 nm in all three cases. The second most common spacing, although not always visually evident in a random population of molecules, is apparent when the data are plotted with appropriately sized groupings. The peak of this distance falls between 25 and 30 nm in all three histograms, with few side projections >60 nm apart. Since the side projections are thought to represent groups of chondroitin sulfate chains, this spacing of 25–30 nm may indicate the distance between the groups of chondroitin sulfate chains that are close enough to condense together to form a single side projection.

DISCUSSION

Since Rosenberg et al. (32, 33) first applied the technique of cytochrome *c* spreading of macromolecules to bovine nasal septum proteoglycans, a number of microscopic studies have been done on cartilage proteoglycans from a variety of sources (2, 4, 5, 20, 25, 28, 34). Recent work in this laboratory has dealt with the involvement of extracellular matrix molecules in the embryonic development of chick with the focus on changes in proteoglycans during cartilage, muscle, and bone development (8–14, 21, 30). This report extends those biochemical and morphological analyses and attempts to characterize the unique muscle proteoglycan by visual observations and correlate these findings with known biochemical characteristics. As a basis of comparison, chick cartilage proteoglycans from culture were further characterized as a standard. In this regard, only D1 molecules were analyzed. The differences in morphology of the two large proteoglycans isolated from the leg muscle of day 11 embryos are sufficient to permit grouping of individual molecules into separate categories based on visual interpretation of qualitative char-

acteristics. The categories could be further analyzed quantitatively to determine variations between the different molecules.

Viewing of spread proteoglycans in the D1 fraction prepared from chick embryonic leg muscle affirms the presence of a unique proteoglycan that has very long side projections. To assess quantitatively these and other molecules, all recognizable spread molecules were grouped into simple categories based on size, shape, and complexity. There is no definitive means of determining from available data the relationship of categories I and II to what is known about the proteoglycans by biochemical analysis. They could represent fragments of larger proteoglycans or they could be images of the dermatan sulfate and heparan sulfate fibroblastic proteoglycans described biochemically as small units with perhaps only one or two glycosaminoglycans (12). On the other hand, categories III and IV, which make up most of the embryonic leg muscle proteoglycans found in the D1 fraction, can be directly correlated with the biochemical characteristics. We identify the category IV molecules as muscle proteoglycan because myogenic cells in culture synthesize proteoglycans that have the category IV morphology of long side projections extending from a core length of ~250 nm. The other image in day 11 embryo leg muscle proteoglycan spreads (i.e., category III) resembles that observed with cartilage proteoglycan whose side projections are usually not extended even though the core length is also in the 200 to 250 nm range, and these molecules are identified as mesenchymal proteoglycan.

Wight and Hascall (36) characterized a large proteoglycan isolated from arterial smooth muscle. Superficially, the skeletal muscle proteoglycan described in this report resembles the smooth muscle proteoglycan as visualized by molecular spreading techniques. The side projection lengths on the smooth muscle proteoglycan average 70–75 as compared with 79 nm for the skeletal muscle proteoglycan. The core protein length of the smooth muscle proteoglycan is shorter, 140 vs. 205 nm. These differences are probably reflected in the hydrodynamic sizes of the two molecules. The skeletal muscle proteoglycan has a Sepharose CL-2B K_{av} of 0.1 (Table I) as compared with 0.31 for the smooth muscle proteoglycan. In addition, the estimated average molecular weight of the chondroitin sulfate present in the skeletal muscle proteoglycan is substantially larger, 70,000 vs. 40,000. Although the two large muscle proteoglycans are not identical in structure, both contain exceptionally long glycosaminoglycan chains that are easily discernable by electron microscopy from those of cartilage proteoglycan.

It must be stated that the biochemical analyses of proteoglycan monomers and glycosaminoglycans are derived strictly from [³⁵S]sulfate-labeled molecules (11, 12, 14) and, therefore, represent the sulfated proteoglycans synthesized by cells over a restricted time frame (6 h). In contrast, the images analyzed by molecular spreading represent all the D1 macromolecules present in the tissue at the time of isolation. In the electron microscopic analysis, there is a 3:1 ratio of category IV to category III molecules (i.e., muscle proteoglycan to mesenchymal proteoglycan), whereas in the biochemical analysis, the synthesis of mesenchymal proteoglycan on day 11 is considerably greater than that of muscle proteoglycan, perhaps as much as fourfold higher (12). The difference in the relative amounts of category III and IV molecules determined morphologically and muscle proteoglycan and mesenchymal proteoglycan measured biochemically may result from the

fact that in the latter only newly synthesized molecules are analyzed and the turnover of the two classes of molecules may be different. Thus, if the mesenchymal proteoglycan is catabolized much more rapidly, the muscle proteoglycan would be expected to accumulate in the tissue relative to the mesenchymal proteoglycan, in spite of its lower amount of synthesis, which would reconcile the biochemical and microscopic observations. Studies of the turnover of these two proteoglycans are in progress.

By visualizing molecules in the electron microscope, we can identify and quantify the two large muscle proteoglycans that have been elucidated biochemically. Separating these two groups by purely biochemical means is difficult if not impossible (12). However, as presented in this report, these molecules can be quantitatively evaluated under the electron microscope as two completely separate groups. Electron microscopic visualization of proteoglycans can be used to compare resident molecules (visualized by spreading) with newly synthesized molecules (analyzed biochemically by radiolabeling). We have shown that synthesis of the large proteoglycans, both muscle and mesenchymal, decreases in older tissues (12), but it has not been determined if either or both of these proteoglycans are catabolized or reside in the tissue long after the cessation of their synthesis. This can be addressed by molecular spreading techniques that examine all the proteoglycans present in the tissue at isolation.

The extension of the glycosaminoglycans of the muscle proteoglycan must be due to inherent characteristics of the molecule, since these differences relative to cartilage and mesenchymal proteoglycans were predicted from the biochemical characterization of glycosaminoglycans from each group. Although the molecules from the cartilage preparations could be grouped similarly to the muscle material, monomers of the category IV type of the leg muscle material are never observed. The fact that the muscle proteoglycan retains its spreading characteristics regardless of the presence or absence of carbon on the grids may imply a rigidity of these glycosaminoglycans that is not present in the mesenchymal or cartilage proteoglycans. As of now, the reason for the unique morphologies is undetermined. It could be due to the differences in the size or sulfation position of their glycosaminoglycans or their frequency of chondroitin sulfate chains along the core protein. Such possibilities can be tested by comparison of the spreading morphologies of proteoglycans of different structure and proteoglycans whose structures are altered chemically or enzymatically. We are now conducting studies of this type.

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