Dynamic Expression of Proteoglycans during Skeletal Muscle Development

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Abstract

Proteoglycans are glycoconjugates composed of a core protein and covalcntly attached glycosaminoglycans. Skeletal muscle produces a number of different types of proteogiycans, and, importantly, the types of proteogiycans made by skeletal muscle vary during muscle development. At early stages of muscle development, large chondroiim sulfate proteogiycans of the PG-M/versican type are produced both in culture and *in vivo*. Localization both by autoradiography of radiolabeled material and by imrnunohistochemistry indicates that the chondroitin sulfate proteogiycans are deposited in a peri cellular region around the muscle cells. Although biosynthesis of these molecules is not detected in mature skeielal muscle, their synthesis is re-initiated during regeneration, which suggests a requirement for these molecules in some early aspect of muscle development. Al later stages of muscle development, small dermatan sulfate proteogiycans are synthesized. One of the ::iajor types of dermatan sulfate proteogiycans in skeletal muscle is decorin, which can bind to collagen and affect fibril formation. Decorin is initially localized in the fibrous connective tissue areas of skeletal muscle, but eventually, at later stages of muscle development, is also found in proximity to the myotubes. Hcparan sulfate proteogiycans are also present in skeletal muscle, and among these are molecules of the syndecan, glypican, and perlecan types. Heparan sulfate proteogiycans are significant because of their involvement in signal transduction of growth factors such as fibroblast growth factor. Evidence indicates that hcparan sulfate proteogiycans play a role in the stimulation of myoblast proliferation by fibroblast growth factor. The exact roles played by other proteogiycans in muscle development are unclear at present. However, proteogiycans have the ability to affect cell adhesion and migration, processes which are important in muscle development. Because of this, it is likely that the changing patterns of proteoglycan biosynthesis during muscle development arc part of and, indeed, influence the orchestrated cellular interactions which are essential for proper muscle formation.

Key words: proteoglycan, extracellular matrix, muscle development, chondroitin sulfate, heparan sulfate, dermatan sulfate.

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Proteoglycans are covalent adducts of carbohydrate and protein which are most often found in the extracellular matrix []]. The carbohydrate component of proteogiycans consists of a family of glycans known collectively as glycosaminoglycans. There arc four major classes of glycosaminoglycans: hyaluronic acid, chondroitin/dermatan sulfate, keratan suifate, and hcparin/heparan sulfate. Most proteogiycans contain a single type of glycosaminoglycan, although some proteogiycans have been shown to possess two classes of glycosaminoglycan [1]. The most well known of these is aggrecan, the large proteoglycan of cartilage, which contains both chondroitin sulfate and keratan sulfate [1]. All glycosaminoglycans are repeating disaccharides and contain carboxylate and/or sulfate, which give these polysaccharides a net negative charge (Fig- 1).

Hyaluronic acid is the only glycosaminoglycan whose reducing terminus is not covalently attached to protein, although protein has been found covalently attached to hyaluronic acid at a different site [2]. It is also the only glycosaminoglycan which does not contain sulfate. Hyaluronic acid is generally larger than other glycosaminoglycans and can have a molecular weight in the millions. The heparin/heparan sulfate class is the most structurally diverse, in that the uronic acid moiety can be glucuronic acid or iduronic acid and the sulfate groups can occur in a variety of positions (Fig. 1). A single heparan sulfate glycosarninoglycan can contain any number of the various

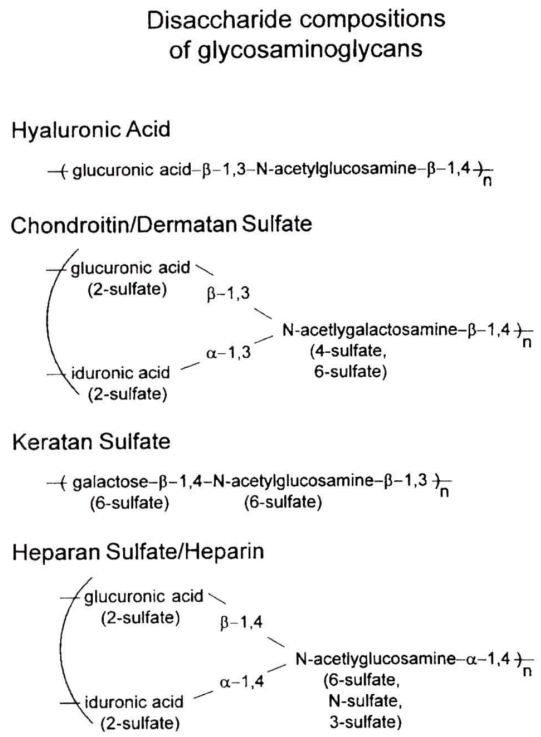

Figure 1. Disaccharide structures of the four major glycosaminoglycans. Each glycosaminoglycan is composed of repeating disaccharides which can have modifications with respect to the identity of the uronic acid moiety. The possible positions for sulfation are indicated in parentheses beneath (he sacchandes where sulfation can be present. It is important to emphasize that a single glycosaminoglycan polysaccharide chain has varying degrees of the different saccharide modifications and vaiy-ing amounts of sulfation in the different possible positions. These variations from the basic disaccharide structure and in the amount and position of sulfation are characteristic of the source of the glycosaminoglycan and are placed non-randomly within the glycosaminoglycan chain.

disaccharides. However, these disaccharides are not randomly distributed along the length of the chain [3]. Many hcparan suifate proteoglycans occur in the cell membrane where they function to bind other molecules through specific sites in the heparan sulfatc. The most well known member of the heparin/heparan sulf'ate class is the anti-coagulant, hep arm. This molecule is a population of heparan sulfate which contains a specific penlasaccharide sequence which allows it to bind to antithrombin [4,5], This binding leads to a greatly enhanced activity of antithrombin to inhibit thrombin and, thereby, inhibit blood coagulation [4, 5]. Keratan sulfate glycosaminoglyeans are most abundant in cartilage and in the cornea, although they are found in other tissues. These glycosaminoglycans tend to be the smallest, with molecular weights of only a few thousand. Lastly, the chondroitin/dermatan sulfate class is composed of two related molecules, chondroitin suifate and dermatan sulfate. Chondroitin sulfate is a repeating disaccharide of glucuronic acid and N-acetylgalactosamine. The amino sugar can be sulfated at either the 4 or 6 position and the uronic acid residue at the 2 position. As with heparan sulfate, a single chondroitin sulfate chain can contain a number of sulfate variations, including different permutations of disulfated disaccharides. The proportion of the various sulfated disaccharides is characteristic of the source of the chondroitin sulfate. Dermatan sulfate is a derivative of chondroitin sulfate in which the uronic acid moiety is epimerized to iduronic acid. Normally, not all of the disaccharides of dermatan sulfate contain iduronic acid, and the ratio of iduronic acid to glucuronic acid is characteristic of the source of dermatan sulfate.

The protein components of proteoglycans, known as core proteins, have recently been subjected to detailed characterization as the techniques of cDNA sequencing have been applied to the analysis of these proteins [6]. Some of these core proteins are very large and have the capacity to harbor many glycosaminoglycans. Others are small and have attachment sites for very few glycosaminoglycans. Some core proteins also contain regions which allow interaction with other molecules or intercalation into cell membranes.

There is a growing body of evidence that proteoglycans can affect several important biological functions and are not simply passive, structural molecules. For example, several studies have shown the potential for chondroitin sulfate proteoglycans to influence cell migration [7-12] and tissue morphogenesis [13-17], both of which are crucial in myogenesis. For example, it is known that the myogenic cells of the limbs arise in the somites and migrate into the limbs [18 and references therein]. This involves not only an extensive cell migration, but also interaction of the migratory myogenic cells with the resident cells of the limb to produce the final morphology of the muscle organ. In addition, as discussed in detail below, certain proteoglycans have been shown to modulate the effects of cytokines [19-21]. Because of the involvement of proteoglycans in biologic processes which are important for muscle formation, the proteoglycans synthesized by skeletal muscle during development should be subjected to detailed analysis. The following is a synopsis of our work in this area and its relationship to relevant studies of others.

Materials and Methods

Radioisotopic labeling in vitro and in ovo

Cultures of myogenic cells were established from day 11 chick embryonic leg muscle [22] and radiolabcled at various times which correspond to different stages of myogenesis. These stages are proliferating myoblasts (day 1), newly fused myotubes (day 3), and contracting myotubes

(day 6). Tn addition, some cultures were treated for 24 hours on day 3 with cytosine arabinoside to eliminate most of the fibroblastic cells. Because these cells continue to proliferate after fusion has occurred, by day 6, the cultures contain a large number of fibroblastic cells, which form a confluent layer between the mature myotubes. In contrast, day 6 cultures which had been treated with cytosine arabinoside contain mature myotubes and a small number of fibroblasts. Cultures were labeled with [Sjsulfate and either j°H]glucosamme or [Hjleucine for 6 hours, during which time incorporation into macromolecular material is linear [23-25].

Chick embryos were radiolabeled *in ovo* at different stages of leg muscle development: day 11, which is prior to the bulk of fusion, day 14, which is shortly after the majority of fusion, and day 17, at which time the muscles have matured. *In ovo* radiolabeh'ng was accomplished by introducing [Sjsulfate through small windows previously cut in the eggshells on day 3 [26]. The eggs were incubated for 6 hours, during which incorporation is linear [26].

Proteoglycan isolation and analysis

Proteoglycans were extracted by standard procedures, which involve extraction of cell culture material or dissected embryonic tissue by stirring overnight at 4 C in a solution of4 M guanidinium chloride, 0.5% CHAPS, 0.05 M sodium acetate, pH 5.8 containing protease inhibitors [25, 26]. Culture medium was processed directly or, in earlier experiments, precipitated with ethanol and the precipitate solubilized in the same solution used for tissue extraction [23]. Proteoglycans were isolated by anion exchange chromatography as previously described [27] with modifications [25,28,29].

Analytical Sepharose CL-2B. CL-4B, and CL-6B columns were prepared and run as described [23-25]. Glycosaminoglycans were removed from proteoglycans by treatment with alkaline sodium borohydride [30]. Identification of the type of glycosaminoglycan was performed by specific degradative treatments [25]. Analysis of the disaccharides in chondroitin/dermatan sulfate was done by thin layer chromatography [31, 32]. Removal of chondroitin/dermatan sulfate for core protein analysis was effected by treatment with chondroitinase in the presence of protease inhibitors [25,33]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described [25, 29, 34]. Immunoblotling in a dot blot or western blot format was done as previously reported [25, 29], Immunostaining of tissue sections [35] and electron microscopy of isolated proteoglycans [25, 36] were performed as described,

Results and Discussion

The earliest studies of glycosaminoglycan synthesis by skeletal muscle cells showed the synthesis of primarily hyaluronic acid and chondroitin sulfate by both myoblasts and newly fused myotubes [37, 38]. These studies, which were performed by radiolabeling primary cultures of chick embryonic leg muscle cells, also showed the synthesis of lesser amounts of heparan sulfate [37, 38]. A subsequent study examined the patterns of glycosarninoglycan synthesis in similar cultures by radiolabeling at various times [39]. These results indicate that, as the myogenic cells proceed from myob lasts to myotubes, there is a proportional increase in the synthesis of heparan sulfate and proportional decreases in the synthesis of chondroitin sulfate and hyaluronic acid [39]. Although these studies provided the first information regarding glycosaminoglycan synthesis by skeletal muscle, the analysis applied only to glycosaminoglycans, rather than the parent proteoglycan molecules.

Our laboratory undertook a study to extend the results of these earlier investigations by examining intact proteoglycans synthesized in primary cultures of chick embryonic leg muscle cells. These data show that myob lasts and newly fused myotubes produce a very large chondroitin siilfate proteoglycan [23-26]. This proteoglycan possesses chondroitin sulfate chains that are *very* long and have an unusually high proportion of 6-sulfated, as opposed to 4-sulfated, chondroitin sulfate [23, 25]. The structure of this proteoglycan is shown diagrammatic ally in Figure 2. Interestingly, the sulfation pattern of this proteoglycan, 80-90% 6-sulfated chondroitin sulfate, is the same as that observed in the earlier studies of glycosaminoglycans [37, 39]. Our studies also demonstrated that contractile myotubes can still synthesize this proteoglycan, since its synthesis is observed in day 6 muscle cultures which have been treated with cytosine arabinoside [23]. However, in untreated day 6 cultures, the major proteoglycans synthesized are small dermatan sulfate and heparan sulfate proteoglycans [23]. Others have also shown that there is an increase in the proportion of heparan sulfate proleoglycans synthesized in older primary cultures of chick embryo muscle cells [40]. Hence, during *in vitro* myogencsis, there is a change from synthesis of primarily large chondroitin sulfate protcog I yeans to primarily small dermatan sulfate and beparan sulfate proteoglycans. This pattern correlates with that reported in the earlier study for glycosaminoglycans [38, 39].

Analysis of the proteoglycans synthesized *in vivo* shows that a similar pattern occurs in the leg muscle and the pectoral muscle of chick embryos [26]. Synthesis of a large chondroitin sulfate proteoglycan was also demonstrated in the embryonic heart [26]. This molecule contains chondroitin sulfate which is similar in size and sulfation pattern to that of skeletal muscle [26]. Also, synthesis of the large chondroitin sulfate proteoglycans in the heart decreases relative to synthesis of small proteoglycans [26], In addition, a similar pattern of proteoglycan synthesis occurs in the skeletal muscle of mice [41], To further examine *in vivo* proteoglycan synthesis in chick embryos, tissue sections of leg muscle from embryos of various ages were subjected to various treatments which degrade specific glycosaminoglycans and then the effects observed both on autoradiographs of tissue sections from embryos radiolabeled

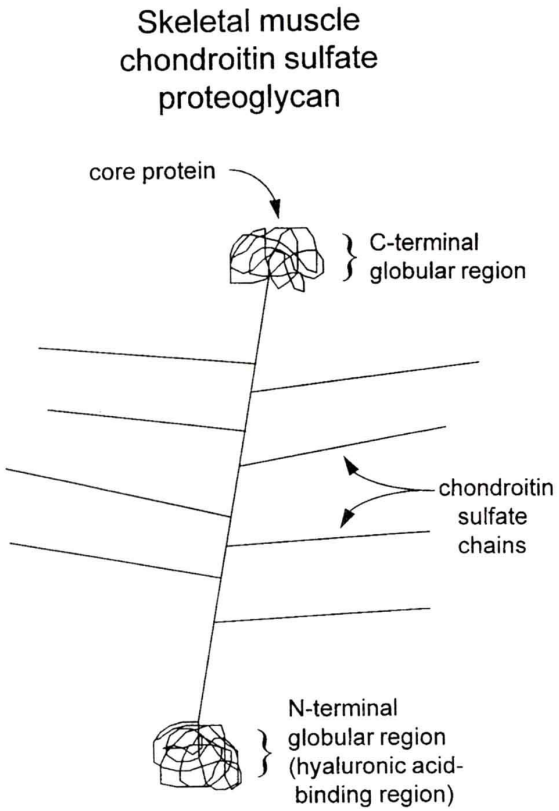

Figure 2. Schematic representation of the large chondroi-tin sulfate proteoglycan synthesized by skeletal muscle. This diagram depicts (he core protein with its amino-terminaf and carboxy-terminal globular regions, which are discussed later in the text. This diagram also depicts the large chondroitin sulfate chains present on the molecule. Not represented are small O-linked and N-linked oligosafcharide.s which have been found on this and other proteog/y-cans [1, 24].

with with [^{3S} S]sulfate and on staining of tissue sections with Alcian blue, a cationic dye which stains glycosaminogly-cans [42]. One result of this analysis is the observation that the majority of the radiolabeled material is localized ex-trace 11 ularly in the matrix surrounding the muscle cells and the fibrous tissue within the muscle organ [42], Moreover, consistent with the biochemical data, there is increased radiolabel in chondroitin sulfate early in myogenesis and increased radiolabel in dermatan sulfate and heparan sulfate later in myogenesis [42J. Interestingly, Alcian blue-positive chondroitin sulfate is present in the muscle after the major period of chondroitin sulfate biosynthesis, which suggests that at least some of the chondroitin sulfate pro-teoglycans are retained in the tissue for some time after their synthesis [42].

In another series of experiments, proteoglycan synthesis was examined in regenerating muscle to determine whether there is re-initiation of the synthesis of pro teogly-cans made during embryonic myogenesis [28]. Two modes of muscle injury were used, cold injury (injury of the muscle with a small metal rod chilled in liquid nitrogen) and partial excision (surgical removal of a small piece of muscle), and two sites were injured, pectoral muscle arjd leg (gastrocncmius) muscle [28]. In the contralateral, un-injured muscle of the adult chicken, only small dermatan sulfate and heparan sulfate proteoglycans are synthesized [28]. In (he regenerating muscle, irrespective of the type of injury or the muscle used, large chondroitin sulfate proteoglycans are synthesized as early as four days after injury [28], The proportion of these molecules among newly synthesized proteoglycans gradually diminishes [28]. Thus, synthesis of large chondroitin sulfate proteo-glycans is not restricted to embryonic skeletal muscle, but can also occur in adult muscle during regeneration. This correlates with other studies which show the re-appearance of other embryonic molecules during muscle regeneration [43-45], In addition, the studies of proteoglycan synthesis in regenerating muscle suggest that the large chondroitin sulfate proteoglyeans function in some early aspect of myogenesis. Nevertheless, whatever their function, there appears to be a general pattern of proteoglycan synthesis m developing tissues from primarily large chondroitin sulfate proieoglycans to mainly small dermaian sulfate and/or heparan sulfate proteoglycans; this pattern has also been reported for tendon [46], liver [47,48], skin [49], and cornea [50].

In addition to sulfated gl y cosam in ogl yean -con tain ing proteoglycans, the nonsulfated glycosaminoglycan hyaluronic acid also shows development-related differ-ences during myogenesis. This was observed in one of the early studies of skeletal muscle glyeosaminogtycans, in which hyaluronic acid synthesis showed a relative de-crease during *in vitro* myogenesis [39] and was sub-sequently reported as part of another study [51], Our laboratory found that the size of the newly synthesized hyaluronic acid decreases somewhat as myogenic cells differentiate in culture. Hyaluronic acid made by myoblasts elutes from Sepharose CL-2B with 60% in the void volume, while only 40% of myotube hyaluronic acid does so (Carrino, D.A. and Caplan, A.I., unpublished observation). Developmental changes in hyaluronic acid may be of importance, since this glycosaminoglycan has effects on *in vitro* myogenesis. For example, when myoblasts are cultured on a substrate of hyaluronic acid, they fail to fuse and continue to proliferate [52]. This effect is not observed if the cells are cultured on chondroitin sulfate and is reversed if cells cultured on hyaluronic acid are subcultured onto a standard gelatin substrate [52], This set of experiments correlates with the findings that myo-genesis by C3H/10T1/2 cells is inhibited when the cells are cultured on an extracellular matrix which has a high ratio of hyaluronic acid to chondroitin sulfate, while matrices with a high ratio of chondroitin sulfate to hyaluronic acid are permissive for myogenesis [53]. It has also been shown

(hat cultured myoblasts contain surface coats whose presence depends on hyaluronic acid and these coats cannot be detected after fusion of ceils into myorubes [54]. Nevertheless, skeletal muscle of mature rats contains hyaiuronic acid, as does cardiac muscle, but, interestingly, hyaluronic acid is not detected in smooth muscle [55].

The recent efforts in sequencing proteoglycan core proteins [1, 6] has allowed the identification of the different Species of proteoglyeans in skeletal muscle. For instance, the large chondroitin sulfate proteoglycan produced early in rnyogenesis has been identified as a proteoglycan known either as versican [56] or PG-M [57], This identification *is* based on three criteria. First, monoclonal antibodies which react with the core protein of PG-M/versican also react with the core protein of the skeletal muscle chondroitin sulfate proteoglycan [25,35], Second, the patterns of peptides produced by cyanogen bromide are the same for the core proteins of PG-M/versican and the skeletal muscle chondroitin sulfate proteoglycan [25]. And third, both PG-M/versican and the skeletal muscle chondroitin sulfate proteoglycan give the same images in the electron microscope after rotary shadowing of isolated molecules [25]. These images are of molecules with core prolcins which have either a single globular region at each end or a globular region at only one end {[25] and Fig. 3). These images are consistent with the deduced amino acid sequence for the PG-M/versican core protein, which predicts a single globular region at each end [56, 57], and provide direct confirmation for the prediction. These images differ from those for the core protein of aggrecan, the large chondroitin sulfate proteoglycan of cartilage, which has a pair of globular domains at one end and a single globular domain variably present at the other [58]. This pattern correlates with that predicted for the aggrecan core prolcin from its deduced amino acid sequence [59]. The globular domains at the ami no-termini of these molecules is the hyaluronic acid-binding region, a domain which allows these proteoglycans to bind non-covalently to hyaluronic acid and thereby form extremely large supra-molecular aggregates [1]. The interaction between the hyaluronic acid-binding region and hyaluronic acid is stabilized by a small glycoprotein known as link protein [1]. Results from our laboratory indicate that the large chondroitin sulfate proteoglycan of skeletal muscle contains a functional hyaluronic acid-binding region [24]. Hence, based on the above criteria, the large chondroitin sulfate proteoglycan of skeletal muscle is a member of the PG-M/versican class of proleogl yeans.

The recent findings of several splice variants for the core protein of PG-M/versican [60-64] suggest the possibility for development-related alterations in the core proteins of skeletal muscle chondroitin sulfate proteoglycans. Another possible, but as yet untested source of developmental alterations is within the fine structure of the chondroitin sulfate. Our laboratory has used monoclonal antibodies to various chondroitin sulfate epitopes to show that these epi topes are distributed along the length of aggrecan chondroitin sulfate in a non-random fashion [65]. This indicates that there may be subtle structural alterations within the chondroitin sulfale chains, analogous to those for heparan sulfate [3]. This notion of chondroitin sulfate structure contrasts with the widely held view of chondroitin sulfate as a monotonous repeating disaccharide with sulfate groups largely randomly placed at either the 6 position or 4 position of the N-acetylgalactosamine residues. There is also evidence that the expression of the chondroitin sulfate epitopes changes during chondrogencsis [66] and during skin development [67], Although iinmunostaining of chick wings at stages much earlier than those examined in our study [35] showed only faint staining with these antibodies [68], it may still be useful to examine those stages where

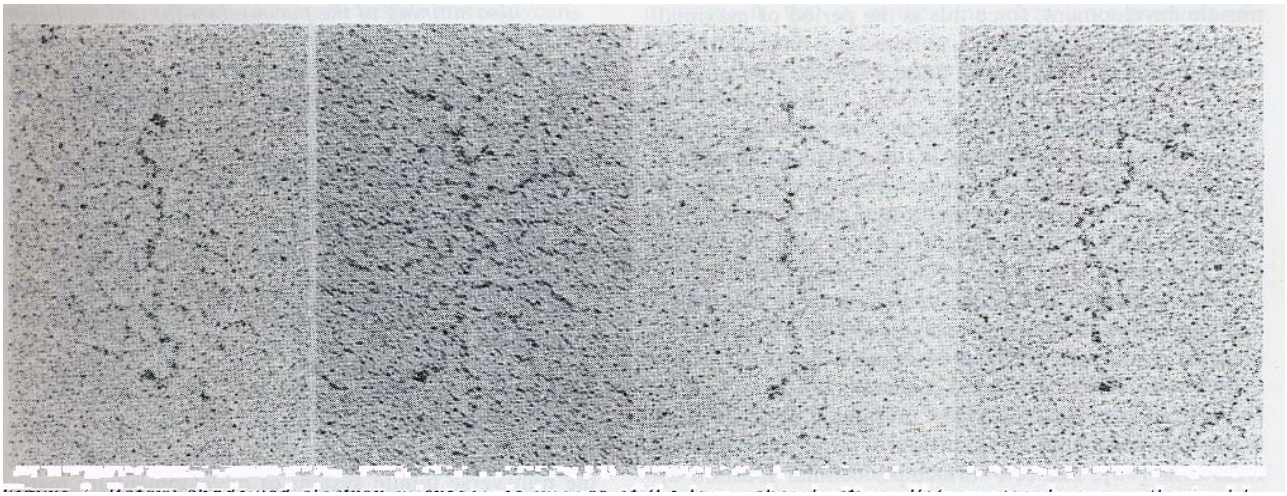

Figure 3. Rotary shadowed electron microscopic linages[1] oj the large chondroitin sulfate proteoglycan svnthesiied by skeletal muscle. The molecules shown were isolated by onion exchange chromatographyfrom the medium of day 3 muscle cut lures. These images are similar to those previously published by our laboratory [25] and show a central filament (core protein) with arms (chondroilin sulfate chains) exlendingfrom it. The core protein contains a globular region either at each end or at only one end.

PG-M/versican is known to be present in the developing muscle ([35]; see below).

One of the same monoclonal antibodies used to analyze the core protein of the skeletal muscle chondroitin sulfate proteoglycan was also used to examine its localization in developing muscle-This study was facilitated by the identification of an anatomic marker at a precise proximal-distalievel in the chick leg j"69]. Because muscle development occurs in a proximal-distal gradient, it is important that the localization analyses be performed at the same site in embryos of different ages, so that any differences can be attributed to development rather than site-related differences [35]. The results of these immunlocalization studies indicate that, at earlier stages, the chondroitin sulfate proleoglycans are located in pericllular regions around the myotubes and arc absent from the fibrous connective tissue regions between the bundles of myotubes ([35] and Fig.4A). With increasing development, PG-M/versican proteoglycans appear in the fibrous connective tissue areas ([35] and Fig. 4B). Our interpretation of these observations is thai there are two distinct PG-M/versican proteoglycans, one made by skeletal muscle cells, which is present early in myogencsis, and one made by fibroblastic cells, which appears later [35]. Our laboratory has previously published evidence, from electron microscopic imaging of isolated molecules, for the presence of two large chondroitin sulfate proteoglycans in embryonic skeletal muscle [36]. Moreover, the pericellular location around skeletal muscle cells con-elates with a similar localization ascertained by staining with ruthenium red, a stain which binds to glycosaminoglycans [37]. In this study, it was also shown that the ruthenium red staining pattern is perturbed by digestion of hyaluronic acid [37].

The role played by PG-M/versican in developing muscle is not yet known. As mentioned above, it has been assumed that these molecules function in some early aspect of muscle development, since this is the period of maximal synthesis of these molecules, and because their synthesis is reinitiated in regenerating muscle shortly after muscle injury. Several studies have provided evidence that PG-M/versican is involved in cell adhesion and migration. *In vitro* studies have shown that PG-M/versican can inhibit the adhesion of several cell types, including chick embryo fibroblasts, to different adhesive molecules, such as fibronectin, laminin, and vitronectin [70], Also, inhibition of PG-M/versican synthesis by osteosarcoma cells results in the cells displaying normal *in vitro* adhesive properties, although cell proliferation is unchanged [71]. These effects on *in vitro* adhesive behavior correlate with the exclusion of PG-M/versican from focal contacts [72]. *In vivo,* PG-M/versican has been found to be present in regions within chick embryos which act as barriers to migration of neural crest cells [73]. The cell surf ace/peri cellular location of PG-M/versican in skeletal muscle [35] may be due to its involvement in adhesive activities.

It is mentioned above that myoblasts contain surface coats whose presence is dependent upon hyaluronic aeid

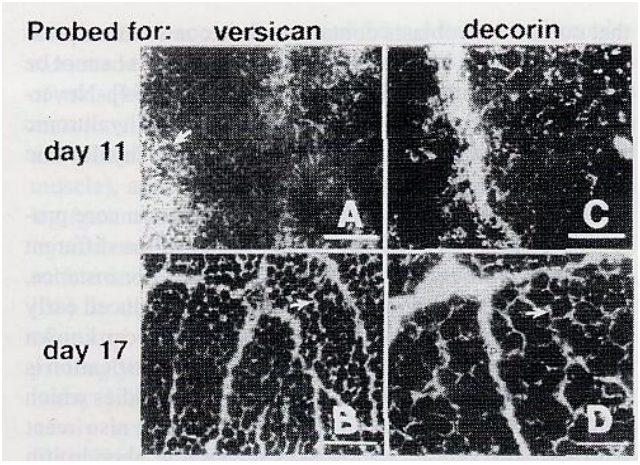

Figure 4. Immunohistochemical localization of she large chondroitin sitifate proteoglycan (PG-M/versican) and of decorin in cross sections of embryonic chick leg muscle on day 10 (stage 36) and day 17 (stage 43) of development. A: Day 10 leg muscle immunostained for PG-M/versican, Strong staining is observed around the myotubes, which are seen in cross section (arrow), while the epimysiutn, which is in the center of the micrograph, stains poorly, if at all; the scale bar is 100 urn. *B: Day 17 leg muscle immiinostainedfor PG-M/versican. Staining is observed in the endomysium around muscle fibers (arrow) and in the fibrous connective tissue areas, such as the perimysium (p); the scale bar is 50 \nm. C: Day 10 leg muscle immunostained for decorin. Strong staining is present in the epimysium (e) and is beginning to be observed in the presumptive perimysium (arrowhead); the scale bar is 100\s.m. D: Day 17 leg muscle immunostained for decorin. The strong staining of the epimysium observed at day !0 is also present at day 17 (e), and there is also staining of the perimysium (p) and the beginning of staining oj the endomysium (arrow); the scale bar is 50* um. *The micrographs in this figure are reprinted from reference 35 with the permission uf the publisher.*

[54] and That the large chondroitin sulfate proteoglycan of skeletal muscle has the capacity to bind to hyaluronic acid in a link protein-stabilized manner [24]. A recent report indicates that link protein is present in skeletal muscle [74]. Thus, the possibility exists that the myoblast surface coats consist of chondroitin sulfate proteoglycans which are maintained in a pericellular location by virtue of link protein-stabilized binding to cell surface hyaluronic acid, although this possibility has not been experimentally addressed. Recently it has been shown by immunolocalization that chondroitin sulfate proteoglycans are present in the transverse tubule system of developmental ly mature skeletal muscle of elasrnobranch, frog, and rat [75]. Biochemical analysis shows two chondroilin sulfate proteoglycans, one very large (molecular weight approximately one million) and one small (molecular weight 280.000)

[75]. Thus, chondroitin sulfate proteoglycans appear to be present in mature muscle, but in a location different from that in developing muscle.

Another proteoglycan present in skeletal muscle is decorin. Decorin is a small dermatan suifate proteogiycan which can bind to collagen fibrils and, in so doing, inhibit fibrillogenesis [1, 6]. Immunostaining with a monoclonal antibody to the core protein of avian decorin [29] shows thai this proteoglycan is expressed in a developmental pattern opposite to that of the PG-M/versican proteoglycan [35]. That is, in the earlier stages of muscle development, decorin is located primarily in the epimysium and, at later stages, it is found there as well as in proximity to the muscle cells [35 and Fig. 4C and 4D). Others have also presented data which show that decorin is present in mature skeletal muscle *in vivo* in a localization pattern similar to that observed in our study [76, 77]. These studies provide evidence for the presence of other dennatan sulfate proteoglycans in skeletal muscle: biglycan, another small dermatan sulfate proteoglycan [76], and a large dermatan sulfate proteoglycan which is related to that found in the cornea [77]. Results from *in vitro* analyses indicate that myotubes synthesize decorin [78]. A particularly intriguing observation is the finding that both pharmacologic and surgical denervation of skeletal muscle leads to enhanced synthesis of decorin [79, 80], This effect was shown to be at the level of transcription of the message for decorin core protein [80] and can be reversed by re-innervation of the muscle [80]. This phenomenon may be related to the observation that solubilization of asymmetric acetylcholinesterase with heparin leads to cosolubilization of decorin [81, 82], Because of decorin's ability to bind to collagen and because of the presence of a collagen tail on the asymmetric form of acetylchnlinesterase, it may be that decorin is part of a complex which contains the acetylcholines(erase, although anchorage of the enzyme to the matrix appears to be due to heparan sulfate proteoglyeans [83, 84].

Skeletal muscle has also been shown to contain the proteoglycan syndecan [85]. Syndecan is a heparan sulfate proteoglycan which, in some circumstances, contains both heparan sulfatc and chondroitin sulfate [Ij. The core protein of syndecan contains a transmembrane domain, which allows the molecule to be intercalated into the plasma membrane [1]. The presence of heparan sulfate proteoglycans in skeletal muscle is of importance, because heparan sulfale protcoglycans have been shown to be involved in signal transduction of growth factors such as fibroblast growth factor [19-21]. That such a process operates in skeletal muscle is shown by recent work with MM14 cells, a mouse skeletal muscle cell line [86. 87]. Under normal conditions, treatment with fibroblast growth factor blocks the differentiation of MM 14 cells from myoblasts to myotubcs [87]. When binding of fibroblast growth factor to cell surface heparan sulfate is abrogated, the growth factor can no longer inhibit differentiation [86, 87]. The presence of syndecan in limb mesenchyme and its gradual disappearance at later developmental stages [85] is consistent with thisproteoglycan's involvement in the proliferative phases of myogenesis.

Syndecan is not the only heparan sulfate proteoglycan made by skeletal muscle. This tissue also contains a heparan sulfate proteoglycan which is attached to the cell surface through a glycosylphosphatidylinositol anchor [88]. Immunolocalization analysis indicates that this molecule resides at the cell surface of skeletal muscle myotubes *in vitro* and in the endomysiuin, but not the perimysium, *in vivo* [88]. The anchorage of this proteoglycan through glycosylphosphatidylinositol makes this a glypican type of heparan sulfate proteoglycan [89]. The amount of glypican synthesized by muscle ceils has been shown to increase during *in vitro* differentiation, but, interestingly, there is no corresponding increase in the level of glypican core protein mRNA, which suggests a post-transcriptional regulation of the increase in glypican [90]. The giypican of muscle cells is released from the cells over time and becomes incorporated into the extracellular matrix [90].

Skeletal muscle contains another heparan sulfate proteoglycan. the large proteoglycan, perlecan, which is present in skeletal muscle basement membranes [91-93]. This localization is as expected based on observations from numerous tissues [91-93]. Evidence from *in situ* hybridization indicates that mature skeletal muscle does not synthesize perlecan [93], which correlates with results showing that at least some of the type IV collagen in skeletal muscle basement membranes is produced by the connective tissue fibroblasts surrounding the muscle cells [94]. Thus, the connective tissue fibroblasts provide at least some of the components for the skeletal muscle basement membranes. Immunohistochemislry of developing skeletal muscle shows that basement membrane heparan sulfate proteoglycans (and also laminin) are not present around individual myotubes at early stages of myogenesis, that is, at the time when large amounts of PG-M/versican are found in the muscle, but rather are observed at later stages, when synthesis of PG-M/versican has declined [35].

It is clear that skeletal muscle cells produce a wide diversity of proteoglycans and that the types of proteoglycans synthesized by skeletal muscle change during muscle development There is increasing evidence that proteoglycans are not only passive structural moieties, but can also affect cell behavior [95-99], Hence, the next area for exploration with respect to skeletal muscle proteoglycans is to ascertain their roles in muscle development. The possible involvement of heparan sulfale proteoglycans in muscle cell proliferation has been established [86. 87]. It has also been shown that a hereditary muscle weakness in chickens correlates with elevated synthesis of decorin at a specific stage of embryonic development [100]. It is not known how decorin is involved in the phenotype, if at all, but this model represents a viable potential to elucidate the role of decorin in muscle development. In another study, muscle cells in culture were treated with chlorate, an agent

which inhibits sulfation of glycosaminoglycans [101]. This treatment causes inhibition of several components of muscle phenotypic expression, such as fusion into myotubes and production of acetylcholinesterase [101], The inhibitory effect of chlorate can be abrogated by culturing the cells on an exogenous extracellular matrix [101]. Taken together, these data indicate that an extracellular matrix is necessary for later events of muscle phenotypic expression [101]. Our laboratory treated cultures of chick leg muscle cells with p-xyloside, a compound which perturbs synthesis of chondroitin sulfatc proteoglycans [102]. Although, as expected, p-xylosidc causes profound effects on the chondroitin sulfate proteoglycans, and some interesting results were obtained regarding proteoglycan synthesis, there is no effect on *in vitro* myogenesis [102]. Because the effects of p-xyloside in other developing systems are on tissue morphogenesis [13-17, 103-107] rather than cytodifferentiation [16,108], our interpretation of the results from xyloside treatment of skeletal muscle cultures is that the chondroitin sulfate proteoglycans function in tissue morphogenesis, a phenomenon which is absent from the simplified environment of the monolayer cultures. A role of PG-M/versican in tissue morphogenesis is consistent with its above mentioned effects on cell migration and adhesion. At this poinl, this possibility remains speculative, and a challenge for the future is to establish the roles played by proteoglycans in muscle development.

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