

Skeletal Muscle Satellite Cells: Identification of a Heparan Sulfate Proteoglycan

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Abstract

Skeletal muscle fibers are surrounded by an extracellular matrix. The extracellular matrix is composed of glycoproteins, collagen, and proteoglycans. Proteoglycans have been suggested by different reports to play an important functional role in tissue differentiation. However, an understanding of how proteoglycans modulate skeletal muscle differentiation and the activation of myogenic satellite cells is largely unknown. In the present study, chicken pectoral muscle satellite cells were screened for the synthesis and localization of a heparan sulfate proteoglycan during satellite cell proliferation and differentiation. A heparan sulfate proteoglycan was detected during the proliferative phase of cell growth. After the induction of fusion, the heparan sulfate proteoglycan had both an intracellular and extracellular distribution. Based on the reported function of heparan sulfate proteoglycans as a modulator of basic fibroblast growth factor activity, it is possible that a satellite cell produced heparan sulfate proteoglycan may interact with basic fibroblast growth factor and be a key component in the satellite cell response to basic fibroblast growth factor.

Key words: muscle, satellite cell, heparan sulfate, proteoglycans.

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Postnatal skeletal muscle development is dependent on the proliferation, differentiation, and fusion of myogenic satellite cells into adjacent muscle fibers [1]. Although muscle fiber number does not increase appreciably during postnatal growth [36], dramatic increases in muscle cell size and nuclear content do occur [1]. This increase in satellite cell-derived nuclei is proportional to [30] and a prerequisite for [29] muscle protein accumulation, i.e., muscle growth. In addition to playing a critical role in postnatal muscle growth, satellite cells reinitiate proliferative activity in response to injury. During the repair process, satellite cells become activated, migrate to the damaged area and form new myotubes to replace the injured muscle tissue.

A wide array of extrinsic factors has been shown to regulate the proliferation and differentiation of myogenic cells during both postnatal hypertrophy and repair [14]. These extrinsic factors include basic fibroblast growth factor [13,16,21, 26, 39], platelet-derived growth factors [16,26, 39], transforming growth factor β [12, 21, 23], and insulin-like growth factors [16, 17,26]. Similarly, the extracellular matrix, which is composed of collagens, proteoglycans, laminin, and fibronectin, surrounds the muscle fiber and may selectively regulate certain myogenic processes.

When skeletal muscle proteoglycan synthesis is suppressed, myoblast fusion and appearance of acetylcholinesterase are inhibited [28]. The skeletal muscle extracellular matrix proteoglycan component is dynamic with a precise temporal and spatial regulation of gene expression [19]. Young et al. [40] and Fernandez et al. [19] have shown that during chicken skeletal muscle development, the extracellular matrix early in embryonic development is predominantly chondroitin sulfate-rich and subsequently modified to a chondroitin sulfate, dermatan sulfate, and heparan sulfate matrix. These changes in the composition of the extracellular matrix may result from the state of innervation of the muscle which has been shown to modulate extracellular matrix expression [18].

Heparan sulfate proteoglycans (HSPGs) have been identified on the cell surface of differentiated myotubes [8]. The presence of HSPGs on the cell surface of myotubes has important implications in regard to the differentiation of muscle in response to basic fibroblast growth factor (bFGF). For bFGF to exert an effect on cellular activity, it is dependent upon binding to cellular heparan sulfate [32]. In avian satellite cells, bFGF is a potent stimulator of satellite cell proliferation [15, 26]. However, there are no reported data regarding the presence of a satellite cell-pro-

duced heparan sulfate proteoglycan which might bind to hFGF.

Since the cellular response to FGF is regulated by HSPGs, it was the objective of this study to determine whether avian satellite cells synthesize a HSPG and if they did, to determine the localization of the HSPG. To address this question, we used a chicken skeletal muscle HSPG monoclonal antibody whose staining is closely associated with the myotube cell surface [2].

Materials and Methods

Satellite cell cloning and culture

Satellite cells were isolated from the pectoralis major muscle of White Leghorn chickens according to the method of McFarland et al. [27] as modified from the procedure described by Bischoff [4]. Following plating of the cells and incubation for 24 h, the viable cells were washed to remove cellular debris, and the remaining viable cells were trypsinized from the substratum and placed in the source well of a Quixell robotic cell manipulator (Stocking Co., Wood Dale, IL). Individual cells were then pipetted from the suspension and placed into separate wells of a 96-well cell culture plate containing medium. The individual clones were grown and then passed to larger wells and eventually to cell culture flasks. The cells were then stored frozen in 9.1% DMSO and 20% horse serum in DMEM until use. Clones possessing the fastest growth rates, good cell morphology, and ability to form myotubes were chosen from both lines for these studies.

During cell proliferation, the satellite cells were cultured in McCoy's 5A + 10% chicken serum + 5% horse serum + 20 ng/ml fibroblast growth factor. After the cells reached 65% confluency, differentiation and fusion were induced by low serum-containing medium consisting of DMEM + 3% HS + 0.01 mg/ml porcine gelatin + 1.0 mg/ml bovine serum albumin.

Immunostaining of satellite cell cultures

Heparan sulfate proteoglycan expression was determined at 24 h intervals during proliferation until 65% confluency was reached and for 96 h during differentiation and fusion. This was accomplished with a monoclonal antibody to the chicken HSPG (Fambrough HS-PG 33; Developmental Studies Hybridoma Bank). Cell culture medium was removed from the culture wells and replaced with ice cold fixative (70% ethanol, 10% formalin, 5% acetic acid) for 1 min. After washing the wells, HS-PG 33 was added at a 1:500 dilution for 1 h. Control samples were treated with a preimmune serum in place of the HS-PG 33. After the incubation in the primary antibody, cultures were washed and fluorescein-conjugated goat anti-mouse antibody (Kirkegard and Perry, Gaithersburg, MD USA) was added for 1 h. After washing, the localization of the HSPG was viewed with an Olympus IX 70 microscope and photographed onto Kodak Ektachrome 200 film. Deconvolution of the images was obtained with Vay-Tec Image Analysis software.

ELISA analysis

At 12 h intervals during 96 h of differentiation and fusion, cell culture samples were harvested. Cells were removed from the culture well by gently scraping with a rubber policeman and were disrupted with a dounce homogenizer in HEPES buffer pH 4.2 (0.02 M Hepes, 0.25 M sucrose, 0.001 M EDTA, 0.001 M EGTA, 0.001 M PMSF, 1 μ g/ml leupeptin). The samples were then dialyzed against distilled H₂O and protein concentrations were determined by the Bradford [6] method. Samples were aliquoted at a 10 μ g/100 μ l concentration, lyophilized, and then resuspended in 0.05 M carbonate-bicarbonate buffer, pH 9.8. Ten μ g of antigen were used to coat the wells of a Falcon (Lincoln Park, NJ USA) pro-bind assay plate and incubated overnight at 37°C. After the incubation, the plates were washed twice with PBS-T (PBS pH 7.4 containing 0.05% Tween-20) followed by a wash with distilled water. Primary HS-PG 33 antibody was added to each well at a 1:500 dilution and incubated at 37°C for 2 h. Controls without primary antibody and no antigen were run with each ELISA assay. After the primary antibody incubation, the plates were washed three times in PBS-T and twice with distilled water. Alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma, St. Louis, MO USA) was added at a 1:20,000 dilution and incubated at 37°C for 2 h. The plates were washed three times with PBS-T and twice with distilled water. To each well 100 μ l of the substrate pNPP (p-nitrophenyl phosphate, Sigma, St. Louis, MO USA) were added and the plates were incubated in the dark for 30 min. After the color development reaction was complete, readings were taken at 405 nm in a BioRad (Hercules, CA USA) ELISA plate reader.

Immunoblot analysis

At 12 h intervals during 96 h of differentiation and fusion, cell culture samples were harvested. Cells were removed from the culture well by gently scraping with a rubber policeman and were disrupted with a dounce homogenizer in HEPES buffer pH 4.2. After homogenization of the cells, protein concentrations were determined by the Bradford [6] assay. Twenty μ g of protein were applied to each slot of a Schleicher and Schuell (Keene, NH USA) slot blot apparatus according to the procedure of Velleman [38]. Sample wells containing BSA as the antigen were included on each blot. The blot was then air-dried for approximately 17 h and then placed in blocking buffer solution (3% gelatin in IX TBS [20 mM Tris, 500 mM NaCl, pH 7.5]) for 1 h at room temperature with gentle agitation according to the method described by Velleman [38]. After the blocking step, the blot was placed in HS-PG antibody diluted 1:500 in 1% gelatin in IX TBS for 2 h at room temperature. The blot was then incubated with an alkaline phosphatase conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA USA) second antibody for 2 h at a 1:3000 dilution in 1% gelatin in IX TBS. The resulting blot was washed and developed with Sigma BCIP/NBT (Sigma, St. Louis, MO). After the color development was

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complete, the blot was scanned with a PDI Quantity One Densitometer (Huntington Station, NY USA) and the reflective density values for each band were determined.

Results

Identification of a satellite cell HSPG

The synthesis of a HSPG by chicken myogenic satellite cells was determined by immunoblot and ELISA analysis. Satellite cell and myotube samples were harvested every 12 h after inducing myogenic fusion. The detectable amount of HSPG was relatively low early in the fusion process (Figures 1A and B, and 2). However, as the fusion process progressed, increased levels of HSPG were detectable. Maximal levels of HSPG were detected 48 h after the initiation of fusion (Figures 1A and B, and 2). This pattern of HSPG expression was reproducible in each assay.

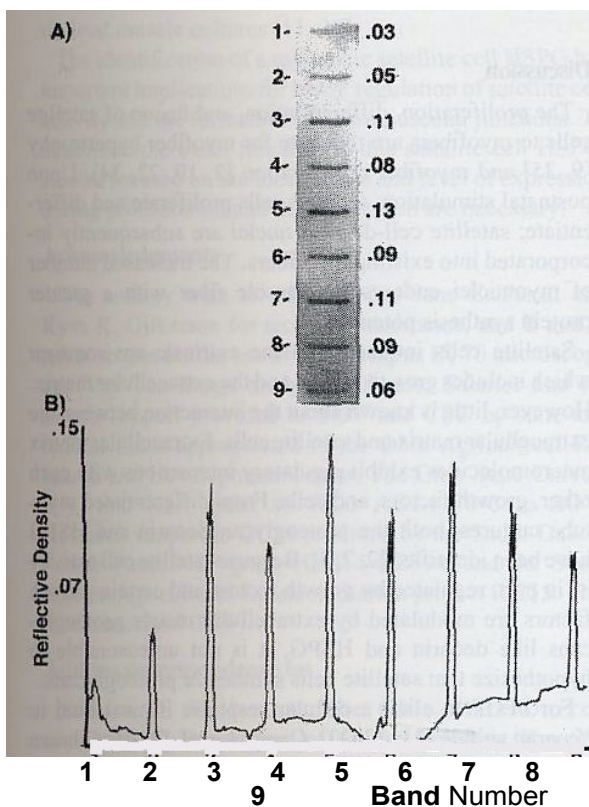


Figure 1. HSPG immunoblot analysis of differentiating and fusing satellite cell cultures. Samples were taken every 12 h for 96 h. A) HSPG immunoblot image, numbers on the left side represent sampling time point with each band number representing a 12 h time interval. Band 1 represents the 0 h time point, 2 the 12 h time point, 3 the 24 h time point, 4 the 36 h time point, 5 the 48 h time point, 6 the 60 h time point, 7 the 72 h time point, 8 the 84 h time point, and 9 the 96 h time point. The numbers on the right side of the figure indicate the reflective density value for each band. B) Illustration of the reflective densities of the HSPG immunoblot in (A).

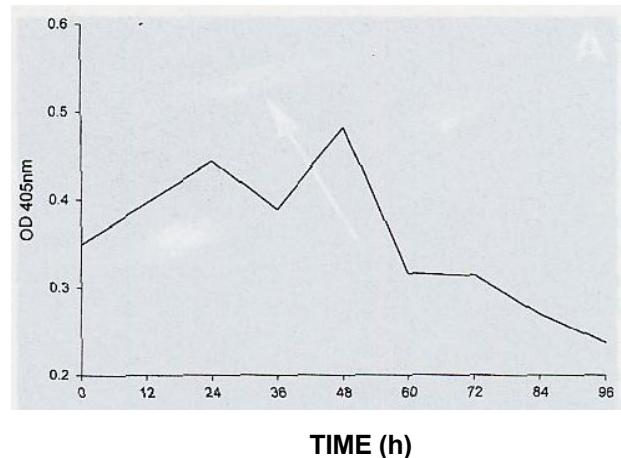


Figure 2. ELISA analysis of differentiating and fusing satellite cell cultures. Samples were taken every 12 h for 96 h.

Presence of a HSPG in proliferating satellite cells

We used indirect immunocytochemical staining with the HSPG monoclonal antibody HS-PG 33 to determine the temporal and spatial localization. The HSPG was detectable in proliferating satellite cells 24 h after plating (Figure 3).

Temporal and spatial localization of the heparan sulfate proteoglycan during fusion

By indirect immunofluorescence the temporal and spatial localization of the HSPG was determined in satellite cell cultures induced to fuse. The fusion process was monitored for 96 h, with samples being harvested every 24 h. The greatest amount of immunoreactivity to the HSPG antibody was observed 48 h following administration of fusion medium. These data correlate with the ELISA and immunoblot. Staining for the HSPG was observed both extracellularly (Figure 4) and intracellularly (Figures 4 and 5). Intracellular distribution was predominantly concentrated around the membrane of the myotube (Figures 4A and 5). Figure 4 illustrates a 48 h myotube after image

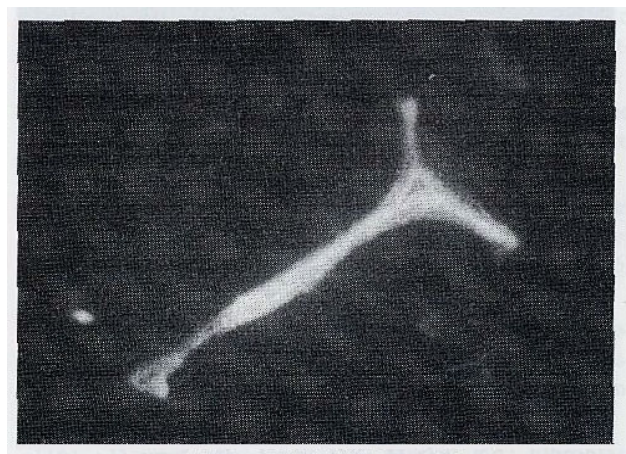


Figure 3. Localization of HSPG in proliferating satellite cells 24 h after the initiation of the cell culture. Magnification 100X.

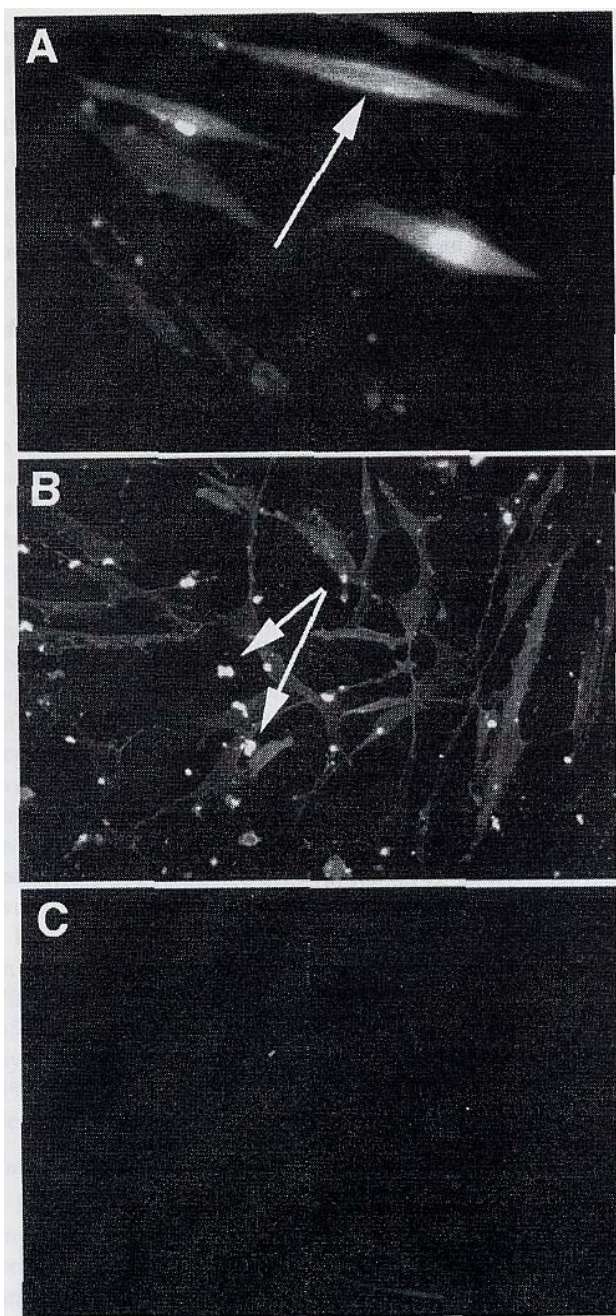


Figure 4. Localization of HSPG in differentiating satellite cells 48 h after the initiation of fusion. A) intracellular localisation of the HSPG; B) extracellular distribution of the HSPG; and C) non-immune serum as the first antibody. In A) and B) the arrowheads indicate areas positive for the HSPG. Magnification 100X.

deconvolution. The image deconvolution highlights the plasma membrane localization of the HSPG. The extracellular distribution was characterized by brightly staining, discretely organized deposits (Figure 4B). These staining patterns were not observed in the presence of non-immune serum (Figure 4C).

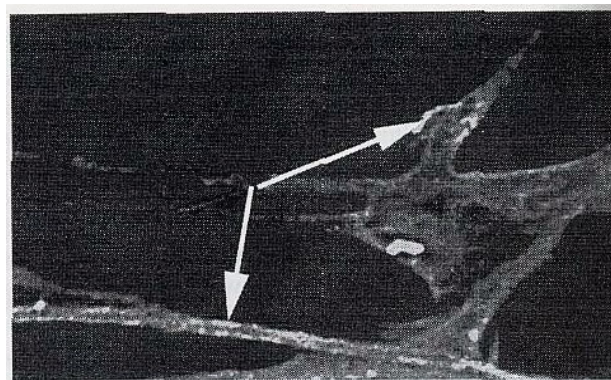


Figure 5. Intracellular localization of the HSPG in a 48 h differentiating satellite cell culture after image deconvolution. The arrowheads highlight the distribution of the HSPG along the plasma membrane. Magnification 100X.

Discussion

The proliferation, differentiation, and fusion of satellite cells to myofibers are requisite for myofiber hypertrophy [9, 35] and myofiber regeneration [3, 10, 22, 34]. Upon postnatal stimulation, satellite cells proliferate and differentiate; satellite cell-derived nuclei are subsequently incorporated into existing myofibers. The increased number of myonuclei endows the muscle fiber with a greater protein synthesis potential.

Satellite cells interact with the extrinsic environment which includes growth factors and the extracellular matrix. However, little is known about the interaction between the extracellular matrix and satellite cells. Extracellular matrix macromolecules exhibit regulatory interactions with each other, growth factors, and cells. From differentiated myotube cultures, both the proteoglycan decorin and HSPG have been identified [2,7,8]. Because satellite cell activity is, in part, regulated by growth factors and certain growth factors are modulated by extracellular matrix proteoglycans like decorin and HSPG, it is not unreasonable to hypothesize that satellite cells synthesize proteoglycans.

For bFGF to elicit a cellular response it must bind to heparan sulfate [31,32,33]. Campos et al. [8] have shown the presence of a hydrophobic HSPG on the surface of differentiated myotubes. In the present study, we have identified a HSPG that is first observable during satellite cell proliferation. During the proliferative phase, this HSPG is localized intracellularly. In contrast, in differentiating cells the localization is both intracellular and extracellular. The intracellular localization is predominantly at the myotube cell surface. In a study by Bayne et al. [2], the HS-PG 33 monoclonal antibody showed a surface organization congruent to the acetylcholine receptor. Acetylcholine receptors have been identified in both proliferating and differentiating satellite cells [25]. Both HSPG and acetylcholine receptors have been shown to colocalize at the neuromuscular junction in differentiated myotube

cultures [5]. Furthermore, on muscle fibers, satellite cells are more prevalent near myoneural junctions [20, 24]. It is possible in satellite cells that there is a colocalization of acetylcholine with HSPGs.

The extracellular distribution of HSPG immunoreactivity in the differentiating satellite cells was rather unusual in appearance. However, during both proliferation and differentiation, the satellite cells are migrating across a gelatin coated surface. It is possible that, during the cell migration process, cell surface components are left behind on the gelatin, which results in the HSPG immune reactivity. A second possibility is that the satellite cells secrete the HSPG into the cell culture medium by a processing mechanism. Campos et al, [8] have shown a lipid-anchored heparan sulfate proteoglycan present in differentiated skeletal muscle cells is released from the plasma membrane into the culture medium. Secreted proteoglycans have been detected in both chicken and ovine primary skeletal muscle cultures [11, 37].

The identification of a myogenic satellite cell HSPG has important implications for bFGF regulation of satellite cell activity and the formation of neuromuscular junctions. To determine the exact function of this satellite cell HSPG, studies focused on the localization and level of expression during proliferation and differentiation are necessary.

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