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Identification and distribution of heparan sulfate proteoglycans in the white muscle of Atlantic cod (*Gadus morhua*) and spotted wolffish (*Anarhichas minor*)

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Abstract

Heparan sulfate proteoglycans (HSPGs) were identified in pre-rigor muscle of two species of cold water fish, Atlantic cod (*Gadus morhua*) and spotted wolffish (*Anarhichas minor*) by biochemical and immunological methods. The distribution was described by immunohistology. Special emphasis was directed to the extracellular matrix (ECM) HSPGs perlecan and agrin. In vivo ^{35}S-sulfate labeling combined with ultracentrifugation in $CsCl_2$, DEAE chromatography and scintillation counting of the eluates, revealed that the content of ^{35}S-labeled PGs was much higher in wolffish than in cod. A considerable proportion of the ^{35}S-sulfated PGs in both species was HSPG, as judged by nitrous acid degradation. HSPG represented, however, a higher proportion of the ^{35}S-sulfated PGs in cod compared to wolffish. Dot blot and electrophoresis/western blot using two different HS-mAbs, 10E4 and HepSS-1 indicated structural differences in the HS-chains of the PGs present. This observation was strengthened by immunohistochemistry, showing that both mAbs detected epitopes in the pericellular area, but the staining patterns were not superimposable. Two different agrin isoforms were identified in both species. Furthermore, in the white muscle of both cod and wolffish, perlecan mAb (A7L6) showed positive staining restricted to the transition between myocommata and myofibers.

Keywords: Heparan sulfate; Fish; Extracellular matrix; Adhesion; Muscle; Perlecan; Agrin

1. Introduction

Heparan sulfate proteoglycans (HSPGs) are members of the proteoglycan family characterized by carrying heparan sulfate (HS) side chains. HS-chains consist of alternating hexuronic acid and glucosamine residues and are known to be structurally heterogenous due to variability in sulfation and epimerization along the carbohydrate backbone. The specific structural properties of the HS-chains determine interactions with a variety of extracellular ligands, growth factors and enzymes (for review see Stringer and Gallagher, 1997; Belting, 2003).

The HSPGs are found in all mammalian organs and tissues that invariably produce more than one HSPG species (Kjellén and Lindahl, 1991). They are located abundantly on the cell surface as syndecans and glypicans, and in basement membranes as perlecan, agrin and type XVIII collagen. HSPGs are involved in various biological processes. Examples are cell adhesion, migration and proliferation, tissue differentiation and organization of extracellular matrix (ECM) (for review see Iozzo, 1998; Dunlevy and Hassel, 2000; Tumova et al., 2000; Nakato and Kimata, 2002; Belting, 2003). The ability of cells to degrade HS is a key factor for the basal membrane break down processes involved in cell motility and metastasis (Kure et al., 1987; Nakajima et al., 1988).

Despite the progress concerning the important role of HSPGs for muscle structure, growth and differentiation in mammalia (for review see Erickson and Couchman, 2000; Iozzo, 2001;

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Velleman, 2002), very little information is available on HSPGs in fish muscle. The muscle fibers of fish are, in contrast to mammalian muscle fibers, arranged as myotomes in concentric circles, and joined head to tail by connective tissue (myocommata). Each muscle fiber is surrounded by endomysium, as in mammalia. Lack of adhesion and splitting of the ECM (gaping) is a major quality problem for the fish industry, as it leads to poor presentation of the fish fillets due to gaps in the muscle, and consequently lower prices.

The HSPGs perlecan (Brandan, 1994; Eggen et al., 1997) and agrin (Ruegg et al., 1992) have been found in the ECM of mammalian skeletal muscle.

Perlecan is reported to play a role in cell adhesion and provides strength to the ECM by interactions with numerous extracellular macromolecules including collagen, laminin, nidogen and fibronectin (Isemura et al., 1987; Battaglia et al., 1992; Bai et al., 1994; Belting, 2003; Iozzo, 1994). In the nematode *Caenorhabditis elegans*, mutations in the *unc-52* gene, the orthologue of the mammalian perlecan gene, lead to disruption of sarcomeres and caused detachment of body wall muscle (Rogalski et al., 1993). Furthermore, the core protein of perlecan in the basement membrane is shown to interact with endothelial cells through β1 and β3 integrins, an interaction modulated by HS (Hayashi et al., 1992).

Structure function studies of agrin have shown that this HSPG binds to several proteins of the cell membrane and the ECM, such as laminins (Denzer et al., 1997) and α-dystroglycan (Gesemann et al., 1998). The amount of agrin in non-synaptic regions changed in models for animal dystrophies (Eusebio et al., 2003).

Pfeiler (1991, 1998) and Pfeiler et al. (1991) reported after series of cellulose acetate electrophoresis that different fish larvae (leptocephali) contained HS in addition to variants of chondroitin sulfate, keratan sulfate and hyaluronan (Pfeiler et al., 2002). A 300-kDa HSPG has been shown to be present in goldfish *anterior optic tectum* (Su and Elam, 2003). Furthermore, we have recently detected HS-chains in cod and wolffish muscle by biochemical methods after digestion of the protein core with papain (Tingbo et al., 2005). Hence, to our knowledge, little has been done to study HSPGs in skeletal muscle ECM of post-metamorphic teleost fish. In the present study, the composition and distribution of HSPGs in pre-rigor muscle of two species of cold water fish, Atlantic cod and spotted wolffish, were investigated by biochemical and histological methods. Atlantic cod and spotted wolffish were selected due to known differences in the propensity to gap (Ofstad et al., in press). A special emphasis was directed on the ECM HSPGs perlecan and agrin.

2. Materials and methods

2.1. Radiolabeling and sampling

Wild caught Atlantic cod (*Gadus morhua* L.), kept in cages while fed, and farmed spotted wolffish (*Anarhichas minor* O.) of average mass 1.8 and 1.7 kg, respectively, were used to study the composition of sulfated proteoglycans in fish skeletal muscle. ^{35}S-sulfate was injected intraperitoneally (1.5 mCi per kg fish) into 3 fish of each species. The fish were killed after 72 h by a sharp blow to the head, first being anaesthetized with 0.05% benzocaine. The radiolabeling trial was repeated with 3 independent individuals of each species.

2.2. Extraction and fractionation of HSPG

Equal amounts (wet weight) of muscle samples from the white muscle beneath the dorsal fin of three individuals of each species were powdered in liquid nitrogen, pooled and incubated over night at 4 °C in 4 M guanidine-HCl in 0.05 M Na-acetate buffer, pH 6. The solid-to-liquid ratio was 10 g tissue to 150 mL extraction buffer. The protease inhibitors 100 mM 6-aminohexanoic acid, 10 mM Na_2EDTA, 10 mM *N*-ethylmalemide and 1 mM phenylmethylsulfonylfluoride were added to the guanidine extraction buffer. The extracts were clarified by centrifugation for 30 min (Beckman centrifuge, JA-14 fixed-angle rotor head, 14 000 rpm, 4 °C).

The extracts were subjected to density gradient ultracentrifugation after addition of $CsCl_2$ to a starting density of 1.37 g/mL. The centrifugation was carried out at 140 000 g for 48 h at 15 °C in a Beckman centrifuge (Optima L-80, fixed-angle VTi50 rotor head) using polyallomer quick seal centrifuge tubes (Opti-seal 25 × 86 mm, Beckman, Fullerton, CA). After ultracentrifugation, the tubes were punctuated in the bottom. 3 fractions, each of 10 mL, were collected from each tube and marked D1, D2 and D3. D1 represented the bottom fraction of the tube with the highest density; D2 was the middle fraction, whereas D3 was the top fraction with the lowest density. The sticky surface layer was discarded. The density of the fractions was determined using a 300 μL pipette as a pycnometer. The different fractions were dialyzed against distilled water and lyophilized.

2.3. Characterization of HSPG

2.3.1. Radioactivity

To follow the recovery of ^{35}S-sulfated HSPG during fractionation, the content of ^{35}S-sulfate was measured after extraction in 4 M guanidine-HCl, and in the D1, D2 and D3 fractions after ultracentrifugation (results not shown). Furthermore, radioactivity was measured in the eluates after DEAE ion-exchange chromatography of D1, D2 and D3 samples, treated or non-treated with nitrous acid (see below). The radioactivity (counts per minute) was measured by scintillation counting in a scintillation counter (WinSpectral 1414 Liquid Scintillation Counter, Wallac, Turku, Finland) after addition of scintillation fluid (Insta-Gel II, Packard Instruments B.V. Chemical Operations, Groningen, Netherlands) in sample-to-fluid ratio of 1 : 2.

2.3.2. Chemical analyses

Before further analyses were performed, the content of protein in the D1, D2 and D3 fractions obtained after ultracentrifugation, was estimated with a Bio-Rad assay (Bio-Rad