Sulfated polysaccharides of brown seaweed *Cystoseira canariensis* bind to serum myostatin protein.

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Summary

Natural sulfated polysaccharides (SPs) derived from brown seaweed comprise a complex group of macromolecules with a wide range of important physiological properties. SPs have been shown to bind and directly regulate the bioactivity of growth factors and cytokines such as basic fibroblast growth factor, interferon, various enzymes and transforming growth factor. Myostatin is a member of the transforming growth factor-β (TGF-β) family that acts as a negative regulator of skeletal muscle mass. In this work we demonstrated that SPs isolated from the brown seaweed *Cystoseira canariensis* bind to the myostatin protein in serum.

Key words: affinity, *Cystoseira canariensis,* serum myostatin, sulfated polysaccharides.

Introduction

Sulfated polysaccharides (SPs) exhibit various physiological activities in mammalian systems and affect many biological processes. For instance, SPs affect the interactions between cells and their surroundings and are crucial factors for the development and integrity of organisms (Lindhal et al. 1998; Varki et al. 1999). Also, fibroblast growth factors (FGF) involved in the control of cell growth, migration, and differentiation, and are thought to mediate their biological effects via SPs -dependent interactions with cell surface FGF receptors (Friesel and Maciag, 1995). SPs bind to various proteins and might protect them from proteolytic enzymes (Yoneda et al. 2000), and control growth factor distribution in some tissues (Chang et al. 2000; Kreuger 2001).

Myostatin is a member of the transforming growth factor-β family that acts as a negative regulator of skeletal muscle mass (McPherron et al. 1997). The importance of myostatin as a regulator of skeletal muscle mass has been well established (McPherron & Lee, 1997; Kambadur et al. 1997; Grobet et al. 1997; Zimmers et al. 2002). Like other transforming growth factor-β family members, the myostatin protein is produced as a precursor protein that contain a signal sequence, an N-terminal propeptide and C-terminal domain that is the active ligand (McPherron, Lawler and Lee, 1997; Zimmer et al. 2002). It was recently demonstrated that myostatin circulates in serum as part of a latent complex (Zimmers et al. 2002). Proteolysis between the propeptide and C-terminal domain releases fully active myostatin (McPherron et al. 1997). Both sub-units, C-terminal and N-terminal domains form disulfide-linked dimmers (McPherron et al. 1997). Hydroxamates-based inhibitors of metalloprotease provoke myotube hypertrophy via reduction of proteolytic maturation of fully active myostatin peptide (Huet et al. 2001).

In humans and animals, myostatin sequences are identical in the biological active C-terminal portion of the molecule (Lee & McPherron, 2001). Using monoclonal antibodies, Hill et al. (2002) isolated the native myostatin

complex from human serum. It was demonstrated that circulating myostatin is bound *in vivo* to at least two major proteins: myostatin propeptide and follistatin-like related gene (Hill et al. 2002), suggesting that myostatin propeptide and follistatin-like related gene are major negative regulators of myostatin *in vivo*. In addition, serum follistatin, an activin-binding protein, interacts directly with myostatin and inhibits its activity (Hill et al. 2002). In the process of purification of follistatin, Nakamura et al. (1991), noticed that follistatin had such a high affinity for SPs, including dextran sulfate and heparin, that it was unable to be eluted from the sulfated matrices even with a 0.5M NaCl.

Because of the apparent affinity of follistatin to SPs (Nakamura et al. 1991) and its natural complexation with myostatin protein (Hill et al. 2002), we postulated that natural SPs might modulate heparin-binding myostatin protein.

In this work we demonstrate that the myostatin protein binds to SPs isolated from a purified extract of the brown seaweed *Cystoseira canariensis*.

Material and Methods

Plant material

The fresh plants of *Cystoseira canariensis,* a marine plant indigenous to the Canary Archipelago, were collected from sublittoral rocks on the cost of Gran Canaria and maintained in culture at the Technological Institute, Las Palmas, Spain. The plant material was harvested, washed with fresh water to remove salt, dehydrated and freeze dried. Dried plant samples are milled and subjected to multiple stage extraction. The freeze dried plant (20gr) was cut in small pieces, suspended in 500 ml of 0.1 M sodium acetate buffer (pH 6.0) and incubated at 60 °C for 12h. The incubation mixture was then centrifuged at 500 rpm for 5 minutes at 5^{o}C and the supernatant saved. The residue was incubated with 300ml of distilled water:ethanol (80:20v/v) for 5 h at 40^{o}C, centrifuged again at 500rpm for 5 minutes at 5^{o}C, and the two supernatants were combined. SPs in solution were precipitated with cold ethanol and the mixture was centrifuged at

5000rpm for 20 min at 5° C. The SPs in the pellet were washed with 50 ml cold absolute ethanol and centrifuged at 5000rpm for 5 minutes at 5^{o}C, and the final precipitate was freeze dried. The crude SPs extract was further purified according to method described by Mulloy et al. (1994), and were quantified following the method described by Farndale et al. (1986) and by the phenol-H_2SO_4 reactions of DuBois et al. (1956).

Serum proteins Chromatography

Serum proteins chromatography was performed following the method first described by Lee and McPherron (2001) with the modifications described below.

Blood samples were collected through a venous catheter into tubes with a phosphate buffer (pH 7.2) containing 10μg/ml leupeptin and 1mM EDTA, centrifuged at 1,000 rpm for 10 minutes to obtain serum plasma. The supernatant (serum plasma) was loaded onto a Sepharose column prepared with 1g SPs isolated from *Cystoseira canariensis*. Preliminary SPs were dissolved in 50 mM Tris-phosphate buffer (pH 7.2) and mixed carefully with Sepharose at 45^{o}C. The mixture was loaded to 10x1.5cm chromatography column and washed overnight with the same buffer. Proteins were eluted from Sepharose-SPs column with 50 mM Tris, pH 7.4/200 mM NaCl, controlled with an UV detector at 280nm. When the UV detector indicated that there were no more proteins eluted with 50 mM Tris, pH 7.4/200 mM NaCl, the elution was continued with the same buffer, except that NaCl was increased to 600mM. The UV detector indicated that more proteins were eluted from the column that were bound to the Sepharose+SPs column. The two fractions eluted with 200mM and 600mM NaCl were collected separately, freeze dried, subjected to SDS-PAGE and Western blot immuno-electrophoresis, and probed with antibodies raised against myostatin peptide.

SDS-PAGE and Western blot protein immuno-electrophoresis

SDS-PAGE was performed using a 10 to 20% acrylamide gradient gel (Laemmli 1970). Western blot protein immuno-electrophoresis was performed as previously described by Wehling et al. (2000). Proteins eluted from different

fractions were prepared in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reducing buffer (80 mM Tris-HCl pH 6.8, 0.1 M dithiothreitol, 70 mM SDS, 1.0 mM glycerol). Samples were boiled for 1 minute, then centrifuged at 12,000 x *g* for 1 min. The supernatant fraction of each sample was removed and used to determine protein concentration by measuring absorbance at 280 nm. Homogenates containing 100 µg of total protein were separated on 10-20% linear gradient SDS-PAGE gels. Proteins were electrophoretically transferred onto nitrocellulose membranes while immersed in transfer buffer (39 mM glycine, 48 mM Tris) (Burnette, 1981). After transfer, membranes were placed in a buffer containing 0.5% Tween-20, 0.2% gelatin, and 3.0% dry milk (blocking buffer) for at least 1 h at room temperature. Membranes were then probed with polyclonal anti-myostatin for 2 h at room temperature. Subsequently, membranes were overlaid with alkaline phosphatase-conjugated anti-rabbit IgG for 1 h at room temperature. Following each incubation, membranes were washed six times for 10 minutes in a buffer containing 0.5% Tween-20, 0.2% gelatin, and 0.3% dry milk. Blots were developed using nitroblue tetrazolium and bromo-chloro-indolyl phosphate.

Myostatin antibody

Anti-Human Myostatin Antibodies were raised in bovine against Human Myostatin his-Tagged Fusion Protein (BioVendor Laboratory Medicine, Inc. Palackeho tr. Brno, Czech Republic). The recombinant human myostatin is 100% homologous with human serum myostatin.

Results

Figure 1 shows protein silver stained SDS-PAGE of serum proteins eluted from Sepharose+SPs chromatography columns with buffer containing 200 and 600mM NaCl. Results indicate that 2 proteins with molecular weights of 36-37 kDa and approximately 12.5-13 kDa were eluted with 600mM NaCI. These

proteins were not present in fraction eluted from the same column with 200mM NaCl, indicating that these proteins were bound to Sepharose+SPs. In a control experiment, we found that native Sepharose beads (without SPs) did not retain these proteins suggesting that these proteins bind specifically to SPs (data not shown).

Figure 2 shows SDS-PAGE and Western blot immuno-electrophoresis of native plasma serum (before fractionation), and two protein fractions eluted from Sepharose+SPs column, probed with antibodies raised against myostatin protein. Results indicated that anti-myostatin antibodies cross-recognized the 36-37kDa peptide in total serum protein (Lane 1) and in fraction eluted with 600mM NaCl (Lane 3), while no detectable cross-reaction was observed in proteins fraction eluted with 200mM NaCl (Lane 2).

Next, we incubated the native plasma serum for 4 h at 5^{o}C in a shaker with different concentration of isolated SPs, and centrifuged at 50,000rpm for 2 h at 5^{o}C. The pellets were collected and again subjected to SDS-PAGE and Western blot immuno-analysis.

Figure 3 shows Western blot immuno-electrophoresis pellets obtained after ultra-centrifugation of native plasma serum with 20mg, 40mg, 60mg and 80mg SPs. These results indicate that the pellets of native serum plasma obtained after centrifugation did not cross-react with the myostatin protein (Lane 1). In contrary, pellets of serum plasma proteins obtained after preliminary incubation with SPs, showed positive cross-reaction with anti-myostatin antibody. Cross-reaction was stronger with increasing he concentration of SPs (Lanes 2-5), indicating that SPs-might bind the myostatin complex.

Discussion

The key finding in this study was that that serum myostatin possesses an affinity to SPs isolated from a purified extract of the brown seaweed *Cystoseira canariensis*. The precise nature of interaction of myostatin protein with SPs of *Cystoseira canariensis* is not clear at this moment. It is possible that it is an ionic

interaction between a cationic protein and anionic SPs. The experiments on the binding nature of myostatin with SP are the topic of our future research. It is also possible to hypothesize that SPs bind to follistatin as it was demonstrated by Nakamura et al. (1991) that might be associated with myostatin protein (Hill et al. 2002). Moreover, Nakamura et al. (1991) demonstrated that the presence of *0* – sulfate groups in SPs are required for the binding of follistatin to SPs. SPs in brown seaweed contain fucose units found mainly sulfated at *0*-2, *0*-3, *0*-4 and at 2,3-*0*-disulfate residues (Chevolot et al. 1999). The core region of the fucans is composed primarily of a polymer of α1-3-linked fucose with sulfate groups substituted at the 4 position on some fucose residues (Patankar et al. (1993).

An analysis of the available scientific data indicates that both natural and synthetic SPs possess a variety of important pharmacological and physiological activities. For example, previously it was demonstrated that SPs bind to the angiogenesis inhibitor endostatin (Sasaki et al. (2001). In another study, Kreuger et al. (2001) demonstrated that SPs bind to fibroblast growth factor receptors. Endo-heparin sulfates isolated from several organs were shown to interact with fibroblast growth factor receptors. SPs have been shown to bind and directly regulate the bioactivity of growth factors and cytokines such as basic fibroblast growth factor, transforming growth factor-, lL-7, and interferon (Salek-Ardakani et al. 2000). SPs inhibit the proliferation of fibroblasts and vascular smooth muscle cells (SMC), in part, by binding to and increasing the antiproliferative activity of transforming growth factor-beta (McCaffrey et al. 1992). SPs also show an inhibitory action on the progression and metastasis of malignant tumors, although the precise mechanisms have not been elucidated. Koyanagi et al. (2003) have demonstrated that natural sulfated and oversulfated polysaccharides derived from brown seaweed inhibit tube formation following migration of human umbilical vein endothelial cells. Sulfated fucoidans stimulated the rate of synthesis of thrombospondin, an inhibitor of angiogenesis (Volpert, 2000). Moreover, the presence of sulfate groups in the SPs molecule was specifically required because desulfation of the fucopolysaccharide abolished the observed effects (Vischer and Buddecke (1991).

Natural sulfated polysaccharides fraction isolated from brown seaweed exhibits the same venous anti-thrombotic activity as heparin in rabbits, but with a lower anticoagulant effect (Giraux et al. 1998). Because of its heparin-like structure, it was postulated that fucoidan might modulate heparin-binding angiogenic growth factor activity (Matou et al. 2002; Giraux et al. 1998). The results indicated that heparin and fucoidan can be used as tools to further investigate the cellular mechanisms regulating the proliferation and migration of human vascular cells. Moreover, the data already suggest a potential role of fucoidan as a new therapeutic agent of vegetal origin in the vascular endothelium wound repair. In addition, natural SPs inhibit the proliferation of fibroblasts and vascular smooth muscle cells (SMC), in part, by binding to and increasing the antiproliferative activity of transforming growth factor-beta 1 (McCaffrey et al. 1992). Unlike heparin, which also suppressed intimal hyperplasia, natural SPs (fucoidans) did not cause systemic anticoagulation, thus, may be useful as a non-anticoagulant inhibitor of post-angioplasty intimal hyperplasia.

Adhesion molecules of the selectin family (mainly P- and L-selectin) have been suggested to mediate interactions between platelets, leukocytes and endothelial cells in thrombus formation. The SPs have anticoagulative properties and are also able to bind and block the function of the selectins. Thorlacius et al. (2000) suggest that fucoidan effectively prevents microvascular thrombus formation induced by endothelial damage in arterioles and venules *in vivo*. Bojakowki et al. (2001) found that administration of SPs (fucoidan), which is a P-selectin inhibitor, increases significantly postischemic renal blood flow and may have renoprotective activity.

Collectively, these results indicate that SPs are physiologically active compounds that participate in the regulation of various cellular processes, including myostatin-binding properties that might open a new dimension in muscle growth research. Sulfated polysaccharides are crucial for vital reactions in the body because of their ability to bind various proteins. Given the key role that myostatin has in regulating muscle growth, these compounds could have positive effects on muscle mass via binding to myostatin or follistatin proteins *in*

vivo. Ultimately, these compounds may also prove to be beneficial in the development of drugs to combat certain muscular related diseases.

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