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DIAPHRAGM DYSFUNCTION IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE: ROLE FOR HEPARAN SULFATE ?

Coen A.C. Ottenheijm[1,4,5#], Guido J. Jenniskens[3#], Maartje C.P. Geraedts[1], Theo Hafmans[1], Leo M.A. Heunks[1,2,4], Toin H. van Kuppevelt[3], and P.N. Richard Dekhuijzen[1,4].

Dept. of [1]Pulmonary Diseases, [2]Intensive Care, [3]Matrix Biochemistry at NCMLS, and [4]Institute for Fundamental and Clinical Human Movement Sciences, Radboud University Nijmegen Medical Centre, the Netherlands, and [5]Dept. of Veterinary and Comparative Anatomy, Pharmacology, and Physiology, Washington State University, Pullman, U.S.A.

Corresponding author:

C.A.C. Ottenheijm
Dept of Pulmonary Diseases
Radboud University Nijmegen Medical Centre
PO Box 9101
6500 HB Nijmegen, the Netherlands
Phone: (+31) 24-3614579
Fax: (+31) 24-3610324
email: c.ottenheijm@long.umcn.nl

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* [#]: Both authors contributed equally

ABSTRACT (word count:206)

OBJECTIVES In the present study we used phage display-derived antibodies to investigate the topology of glycosaminoglycan epitopes in the diaphragm of COPD and non-COPD patients. Furthermore, a potential physiological significance of changes in the occurrence of glycosaminoglycan epitopes in COPD diaphragm was studied by determining the overlap in epitope recognition of glycosaminoglycan antibodies and growth factors.

METHODS Diaphragm cryosections from non-COPD (n=5) and COPD patients (GOLD I/II, n=9) were incubated with antibodies directed against heparan sulfate, chondroitin sulfate, and dermatan sulfate epitopes. Antibodies were visualized immunofluorescently. In addition, interference of antibody and growth factor binding to heparan sulfate epitopes was tested.

RESULTS Specific glycosaminoglycan epitopes show increased expression in COPD diaphragm, whereas other epitopes are decreased or unaffected. Interestingly, the anti-heparan sulfate antibody HS4C3, which is directed against a down-regulated epitope, interferes with the binding of hepatocyte growth factor. Three patients with the most severe airway obstruction also demonstrated interference of heparan sulfate antibody A04B08 with hepatocyte growth factor binding.

CONCLUSION Results indicate changes in glycosaminoglycan composition in the diaphragm of patients with COPD. This may affect cellular physiology via alterations in growth factor handling and might be related to reduced levels of contractile protein in the diaphragm of these patients.

Keywords: COPD, diaphragm muscle, glycosaminoglycan, heparan sulfate

INTRODUCTION

Dysfunction of the inspiratory muscles frequently occurs in patients with chronic obstructive pulmonary disease (COPD) [1], and maximum inspiratory pressure is an independent determinant of survival in these patients [2]. Hence, understanding the underlying mechanisms of inspiratory muscle dysfunction is of major clinical importance.

The diaphragm is the principal inspiratory muscle. Several studies have shown adaptations in diaphragm morphology and function in patients with COPD [3-9]. Recently, we have shown that these patients have a diminished diaphragmatic myosin content, which compromises force generation early in the course of the disease (GOLD I/II) [8]. Contractile protein content is the net result of protein synthesis and degradation. Although the activity of the most important intracellular proteolytic system, i.e. ubiquitin-proteasome pathway, is increased in the diaphragm of patients with mild-to-moderate COPD [10], the possible involvement of mediators that affect the rate of muscle protein synthesis has not yet been investigated.

Glycosaminoglycans are linear unbranched polysaccharides, most of which are covalently linked to a protein core to form proteoglycans [11]. Depending on the nature of the glycosaminoglycan-moiety, one can discern heparan sulfate, dermatan sulfate, and chondroitin sulfate proteoglycans. Most proteoglycans are found either on the cell surface (eg. Syndecan and Glypican), or in the extracellular matrix (eg. Perlecan and Agrin) [12;13]. In skeletal muscle glycosaminoglycans are involved in numerous biological processes, notably the orchestration of anabolic and catabolic signaling by unique sulfation patterns on the heparan sulfate molecule (for review see [14]). Heparan sulfate is essential for the activation of individual members of the fibroblast growth factor (FGF) family [15;16], hepatocyte growth factor [17], insulin-like growth factor binding proteins [18], platelet-derived growth factor [19], and transforming growth factor-beta [20]. The dynamic spatiotemporal expression of proteoglycans

and heparan sulfate epitopes provides a micro-environment in which heparan sulfate mediates growth factor activity by creating focal differences in concentration and by facilitating ligand-receptor interactions [21]. Through this modulating effect on growth factors, glycosaminoglycans are instrumental in skeletal muscle regeneration [22-24].

Investigating the exact nature of the involvement of glycosaminoglycans in myopathies has been hampered by a lack of appropriate tools. We have previously generated an array of glycosaminoglycan-specific antibodies, which can be used to detect potential changes in the topological distribution of glycosaminoglycan epitopes [25]. In the present study we have used these antibodies to investigate the occurrence of glycosaminoglycan epitopes in human diaphragm, and possible changes in the diaphragm of patients with COPD. Furthermore, a potential physiological significance of changes in the occurrence of glycosaminoglycan epitopes, particularly heparan sulfate epitopes, in COPD was studied by determining the interference of growth factors and antibodies on the binding of heparan sulfate epitopes.

METHODS

Subjects and pulmonary function testing

Diaphragm muscle biopsies (~150 mg) from the anterior costal mid-belly region were obtained from nine patients (eight men) with and five patients (five men) without COPD during thoracotomy for lung cancer ($T_{1-3}N_{0-1}M_0$, equally distributed within groups). Fresh biopsy specimens were rapidly frozen in liquid nitrogen-cooled isopentane and stored at -80°C. Exclusion criteria included weight loss of more than 10% in the last six months before surgery, prolonged use of corticosteroids, neuromuscular diseases, thyroid diseases and chronic heart failure. General characteristics and pulmonary function data are shown in Table 1. The study was approved by the local ethics committee and informed consent was obtained prior to surgery.

Phage-display derived anti-glycosaminoglycan antibodies

Phage display-derived single chain variable fragment antibodies were obtained as described previously [25;26]. Briefly, antibody-expressing phages were added to glycosaminoglycan-coated tubes, and bound phages were eluted and allowed to infect *Escherichia coli* cells. After overnight amplification, phages were rescued by addition of helper phage, grown, purified, and used for a next round of selection. Following four rounds of selection, individual phages were picked, grown, induced by IPTG, and antibodies were harvested from the periplasmic fraction. Six antibodies were used in the present study; antibodies HS4C3 and AO4B08 against heparan sulfate, IO3H10, IO3H12, and IO4C2 against chondroitin sulfate, and LKN1 against dermatan sulfate (see table 2).

Immuno-fluorescence studies on glycosaminoglycan epitopes

The occurrence of epitopes recognized by the antibodies was assessed on cryosections by means of immuno-fluorescence microscopy. Cryosections (5 µm) were cut from frozen diaphragm specimens, dried and stored at –80°C. Crysosections were rehydrated for 10 min with PBS, blocked with PBS containing 2% (w/v) BSA for 10 min, and incubated with anti-glycosaminoglycan antibodies for 60 min. Bound antibodies were detected with mouse anti-*VSV* monoclonal antibody (P5D4), followed by incubation with Alexa 488-conjugated goat anti-mouse IgG (60 min each). Cryosections were washed three times with PBS after each incubation. Finally, the cryosections were fixed in 100% ethanol, dried and embedded in Mowiol (10% (w/v) in 0.1 M Tris, pH 8.5/25% (v/v) glycerol/ 2.5% (w/v) NaN_3).

To evaluate the specificity of the antibodies, diaphragm cryosections were pre-incubated overnight at 30°C with heparinase-I/-III to digest heparan sulfate (0.02 IU/ml each in 50 mM NaAc/5 mM $Ca(Ac)_2$, pH 7.0), and with chondroitinase ABC to digest chondroitin sulfate/ dermatan sulfate (0.02 IU/ml in 25 ml Tris-HCl, pH 8.0). As a control, cryosections were incubated in the reaction buffer without enzyme. After washing with PBS and blocking with PBS/2% (w/v) BSA, cryosections were incubated with heparan sulfate or chondroitin sulfate antibodies and processed for immunofluorescence as described above. The efficiency of enzymatic treatment was evaluated by incubation with antibodies against glycosaminoglycan-"stubs". For heparan sulfate-stubs antibody 3G10 was used. For chondroitin sulfate-stubs antibody 2B6 was used (both from Seikagaku, Tokyo, Japan).

Optimal concentrations for primary antibodies were determined in titration series on rat peripheral muscle (m. soleus). All stainings in this study were performed using the conditions that yield maximal staining at minimal antibody concentration. Series of non-COPD and COPD cryosections were incubated in parallel (using the same solutions) and analyzed by two independent observers. Intensity scores were rated for entire cryosections (at 200x

magnification; average cryosection ~0.20 cm^2) by comparison with the maximum staining observed in control tissue (see above): ++, very strong; +, strong; +/-, strong with negative areas; ++/-, very strong with negative areas; ++/+, very strong with strong areas; -, negative; -/+, negative with positive areas. The intensity scores of the two independent observers showed a very high level of agreement. Photographs were taken on a Zeiss Axioskop equipped with a Nikon DXM1200 digital camera with ACT1 software (using similar exposure settings) and figures were compiled using Adobe Photoshop software.

To investigate the expression of proteoglycan core proteins, diaphragm cryosections from COPD and non-COPD patients were incubated with antibodies against perlecan (clone 7B5 from Zymed, San Francisco, CA; 1:100 dilution in PBS-B) and decorin (clone 6B6 from Seikagaku, Tokio, Japan; 1:500 dilution in PBS-B). Bound antibodies were visualized as described above. As a control, primary antibodies were omitted.

Involvement of heparan sulfate epitopes in growth factor binding

To investigate the presence and localization of growth factors, diaphragm cryosections from patients with and without COPD were incubated with antibodies against insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF) (Sigma-Aldrich). The antibodies were visualized as described above. The specificity of the anti-IGF-1 antibody was demonstrated by gradually reduced antibody staining after pre-incubating of diaphragm crysections with increasing concentrations of human IGF-1 (data not shown). Similar results were found for the anti-HGF antibody using human HGF.

To study whether the glycosaminoglycan epitopes are involved in the binding of growth factors, we studied possible interference between growth factor and antibody binding to heparan sulfate epitopes, as described before [27]. Briefly, IGF-1 or HGF were added to diaphragm cryosections at a concentration of 10 µg/ ml in PBS/ 0.1% BSA, and allowed to

bind endogenous heparan sulfate epitopes. Next, cryosections were washed and anti-heparan sulfate antibodies were allowed to bind, and both growth factor and antibodies were visualized. Alternatively, anti-heparan sulfate antibodies were pre-incubated on cryosections followed by incubation with the growth factors. Both heparan sulfate epitopes and growth factors were visualized as described previously. We evaluated whether staining intensity was reduced upon pre-incubation with either anti-heparan sulfate antibodies or growth factors. Results were analyzed by two independent observers.

RESULTS

Subject characteristics

Patient characteristics and pulmonary function data are shown in Table 1. COPD patients were classified as mild or moderate COPD according to GOLD classification [28;29]

Immuno-fluorescence studies on glycosaminoglycan epitopes

All anti-glycosaminoglycan antibodies stained the endo- and perimysium in diaphragm cryosections from patients without COPD. Overall staining intensity of the antibodies ranged from very strong without negative areas (HS4C3, AO4B08, IO3H10, and LKN1) to moderate with negative areas (IO3H12 and IO4C2) (figure 1). All antibodies stained the extracellular matrix of the endothelial layer of blood vessels.

In patients with COPD, the anti-heparan sulfate antibody HS4C3 stained less intensely when compared to patients without COPD (figure 1: a1 and a2, table 3). Patients with COPD had negative areas, whereas this was not observed in patients without COPD. In contrast to heparan sulfate-epitope HS4C3, the anti-chondroitin sulfate antibody IO4C2 appeared to stain more intensely with less negative areas in patients with COPD. AO4B08, IO3H10, IO3H12, and LKN1 immunofluorescence was not different between groups.

Treatment of cryosections with Heparinase-I/ -III and Chondroitinase-ABC completely abolished staining of anti-heparan sulfate antibodies and anti-chondroitin sulfate antibodies, respectively (figure 2). Positive staining of heparan sulfate and chondroitin sulfate-stubs indicates the efficiency of the enzymatic treatment.

The representative micrographs shown in figure 3 illustrate that the staining intensity of both decorin (a chondroitin sulfate/dermatan sulfate proteoglycan, present throughout the

extracellular matrix) and perlecan (an extracellular matrix heparan sulfate proteoglycan, present proximal to the sarcolemma) are similar in COPD and non-COPD patients.

Involvement of heparan sulfate epitopes in growth factor binding

To investigate the localization of endogenous growth factors, cryosections were incubated with anti-growth factor antibodies. In non-COPD patients strong staining of endogenous IGF-1 was observed in the cytoplasm of specific fibers (figure 4A-a1). These fibers were identified as type-I fibers by myosin heavy chain isoform typing (figure 4B). In contrast, endogenous HGF was only faintly detectable in the endomysium (figure 4A-b1). Patients with COPD showed a strongly decreased staining of IGF-1 when compared to non-COPD patients, whereas staining of HGF was increased in the endomysium.

To investigate if the antibody-defined heparan sulfate epitopes co-localize with growth factor binding sites, we performed inhibition-studies on cryosections from both COPD and non-COPD patients. Pre-incubation with anti-heparan sulfate antibody HS4C3 decreased the binding of HGF (figure 5, table 4A), but not of IGF-1. Pre-incubation with anti-heparan sulfate antibody AO4B08 did not affect binding of IGF-1. In contrast to HS4C3, pre-incubation with AO4O8 did not affect binding of HGF, except in three patients with COPD (P11, P12, and P14). In a reciprocal inhibition-experiment we investigated the effect of pre-incubation with exogenous growth factors on subsequent binding of HS4C3 and AO4B08. Pre-incubation with HGF inhibited binding of HS4C3 (figure 5, table 4B), whereas pre-incubation with IGF-1 partially inhibited binding of HS4C3 (table 4B). Binding of AO4B08 was not inhibited by pre-incubation with either of the growth factors.

DISCUSSION

The present study is the first to investigate the topology of glycosaminoglycan epitopes in human diaphragm muscle, particularly in patients with COPD. Our results indicate the down-regulation of a specific heparan sulfate epitope, encoded by antibody HS4C3, in the diaphragm of patients with COPD. Interestingly, this down-regulated heparan sulfate epitope appears to be involved in the binding of hepatocyte growth factor. As hepatocyte growth factor is an important modulator of contractile protein synthesis [30], the observed down-regulation of this heparan sulfate epitope may affect contractile protein content in the diaphragm of patients with COPD. Interestingly, these changes already occur in patients with mild-to-moderate COPD (GOLD stage I/II).

Decreased expression of a heparan sulfate epitope in COPD diaphragm

The heparan sulfate epitopes studied here are located in the endo- and perimysium of diaphragm muscle fibers (figure 1). We observed a decreased staining of anti-heparan sulfate antibody HS4C3 in patients with COPD, whereas staining of the anti-heparan sulfate antibody AO4B08 was not different between both groups. These findings suggest decreased expression of specific heparan sulfate epitopes in the diaphragm of patients with COPD, whereas other epitopes appear to be preserved. Figure 3 suggests that the decreased expression of this heparan sulfate epitope is not merely a reflection of decreased expression of extracellular matrix heparan sulfate proteoglycans present proximal to the sarcolemma [14], as perlecan expression appears comparable between both groups.

Preference for growth factor binding of the down-regulated heparan sulfate epitope

Heparan sulfates are required for successful muscle regeneration after injury [24], through binding and modulating the activity of proteins [31;32], in particular growth factors (for review see Jenniskens et al. [14]). The growth factors HGF and IGF-1 are major regulators in muscle regeneration, as they stimulate quiescent satellite cells upon fiber injury and the subsequent expression of contractile proteins [30;33;34]. COPD diaphragm is characterized by an elevated disruption of sarcomeric proteins [5] and an increased proteolytic activity [10]. In general, muscle injury is followed by an inflammatory response and subsequent regeneration. Despite clear proof of injury, no evidence of increased numbers of inflammatory cells has been reported in the diaphragm of patients with COPD [5;35]. Nguyen et al reported lower expression levels of embryonic/ neonatal myosin heavy chains in the diaphragm from COPD patients as compared to non-COPD patients [36]. These findings are suggestive for an impaired regenerative response to injury in COPD diaphragm. A decreased binding capacity for growth factors, due to the decreased expression of specific heparan sulfate epitopes, could negatively affect muscle protein synthesis and regeneration and thus contribute to the loss of contractile protein[8;9] in COPD diaphragm.

To determine growth factor binding to the heparan sulfate epitopes, we investigated possible interference of HGF- and IGF-1-binding with antibodies specific for the heparan sulfate epitopes. We observed a diminished staining for HGF after pre-incubation with HS4C3. Similarly, pre-incubation with HGF reduced staining of HS4C3. Thus, HGF and antibody HS4C3 appear to compete for binding to similar heparan sulfate modifications. Pre-incubation with HS4C3 did not interfere with the binding of IGF-1. However, pre-incubation with IGF-1 and subsequent incubation with HS4C3 reduced HS4C3-staining. Therefore, IGF-1 might bind to heparan sulfate modifications overlapping with, or nearby the HS4C3-epitope.

In contrast to HS4C3, antibody AO4B08 did not interfere with the binding of HGF, except for three patients with COPD (P11, P12, and P14) (tables 4A and B). Interestingly, these three patients had the most severe COPD of the patients studied (FEV_1 = 58 ± 2% vs 83 ± 7% of predicted). It could be hypothesized that the loss of HS4C3 epitopes in COPD diaphragm is maladaptive by reducing the capacity to bind growth factors, and with progression of COPD this effect might be compensated by enhanced growth factor binding to other heparan sulfate epitopes, possibly the AO4B08-epitope. It should be noted, that the diaphragm cryosections were incubated with supraphysiological growth factor concentrations to assure maximal heparan sulfate epitope-binding and competition with the heparan sulfate antibodies. So, caution is warranted when extrapolating these findings to physiological conditions.

Growth factor presence in COPD diaphragm

Although not our primary focus, the present study is the first to investigate the presence of growth factors in the diaphragm of patients with COPD. The decreased staining of IGF-1 suggests reduced presence of this growth factor in COPD diaphragm. This reduced growth factor presence could lead to a diminished level of protein synthesis and might be related to the decreased contractile protein content in the diaphragm of these patients [8;9]. Remarkably, the IGF-1 staining in non-COPD diaphragm was present predominantly in fibers expressing the myosin heavy chain 1 isoform (figure 4B). Previous studies in rat diaphragm did not find this fiber type-dependent IGF-staining [37]. Future studies should address the physiological significance of the observed fiber type-dependent IGF-staining.

In contrast to IGF-1, the presence of HGF appears to be increased in COPD diaphragm. This might be considered as contradictory to the down-regulation of the 'HGF-binding' heparan sulfate epitope encoded by HS4C3. Possibly, the diaphragm increases the local concentration of HGF to counterbalance the reduction of certain heparan sulfate epitopes. Thus, we

hypothesize that in the diaphragm there is a dynamic interaction between growth factors and heparan sulfate epitopes, which may be altered in COPD.

Increased expression of a chondroitin sulfate-epitope

The three chondroitin sulfate epitopes studied here are also located in the endo- and perimysium of diaphragm muscle fibers (figure 1). The staining intensity of one of these chondroitin sulfate epitopes, encoded by IO4C2, is increased in patients with COPD (see figure 1: e1 vs. e2, and table 3). Interestingly, chondroitin sulfate proteoglycans are up-regulated in muscle fibers of patients with Duchenne muscular dystrophy and in regenerating muscle fibers in calpainopathy [38]. However, the expression of decorin, a chondroitin sulfate/dermatan sulfate proteoglycan present throughout the extracellular matrix [14], appeared not to be elevated in COPD diaphragm (figure 3). This suggests that changes in the expression of the chondoitin sulfate epitope encoded by IO4C2 not necessarily results from alternate expression of proteoglycan core proteins, but might reflect changes in the distribution of GAG epitopes. Future studies should establish if, and how, the up-regulated chondroitin sulfate epitope affects the regenerative capacity of the diaphragm in COPD.

Therapeutic implications: a role for glycosaminoglycan mimetics ?

Recently, it was shown that glycosaminoglycan mimetics, synthetic derivatives of dextran, stimulate myogenesis while changing the natural glycosaminoglycan composition, notably heparan sulfate [39]. These glycosaminoglycan mimetics stimulate the regeneration of denervated and crushed skeletal muscles [40;41], as well as prevent most of the damage resulting from acute skeletal or cardiac muscle ischemia [42]. Therefore, these dextran polymers were called RGTA (for ReGeneraTing Agent). We anticipate the potential therapeutical application

of glycosaminoglycans or glycosaminoglycan mimetics in counteracting diaphragm dysfunction in COPD.

In summary, the present study shows that specific glycosaminoglycan epitopes are altered in the diaphragm of patients with COPD. Since these epitopes might be involved in growth factor binding, the functional loss of heparan sulfate may negatively affect the anabolic-catabolic balance and might therefore be related to the reduced levels of contractile protein in the diaphragm of these patients [8].

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LEGENDS TO TABLES

Table 1. Definition of abbreviations: Values are means ± SEM. Definition of abbreviations: BMI = body mass index; FEV_1 = forced expiratory volume in first s; VC = vital capacity; TLC = total lung capacity; D_{Lco}/VA = carbon monoxide transfer coefficient per alveolar volume; Pao_2 = arterial PO_2; $Paco_2$ = arterial PCO_2.

Table 2. Antibody, anti-glycosaminoglycan antibody clone; CDR3, amino acid sequence of the VH complementarity determining region 3 (a major determinant in antigen-recognition); VH, heavy chain germ line family; DP, gene number; GAG, the class of glycosaminoglycan(s) with which the antibody reacts; Ref, references. HS, heparan sulfate; CS, chondroitin sulfate; Hep, heparin, DS, dermatan sulfate.

Table 3. Immunostaining patterns of anti-glycosaminoglycan antibodies. Cryosections of human diaphragm were incubated with anti-glycosaminoglycan antibodies. Bound antibodies were visualized by incubation with fluorescently labelled antibodies. Staining intensity: ++, very strong; +, strong; -, negative. Staining intensity of cryosections displaying intensity differences between areas were scored as follows: +/-, strong with negative areas; ++/-, very strong with negative areas; ++/+, very strong with strong areas; -/+, negative with positive areas.

Table 4. A: Inhibition of growth factor binding by pre-incubation with anti-heparan sulfate antibodies HS4C3 or AO4B08. Cryosections were pre-incubated with HS4C3 or AO4B08, followed by incubation with growth factors IGF-I or HGF. Bound antibodies were visualized. An overall inhibition of HGF binding is seen after pre-incubation with HS4C3, as indicated

by the negative HGF-staining. Pre-incubation of HS4C3 had no effect on the binding of IGF-I. Antibody AO4B08 had no effect on the binding of growth factors IGF-I and HGF, except in patients P11, P12, and P14 (HGF). B: Cryosections were pre-incubated with growth factors, followed by incubation with HS4C3 or AO4B08, and bound antibodies were visualized. An overall inhibition was seen for HS4C3 after pre-incubation with HGF. After pre-incubation with IGF-I, HS4C3 was slightly inhibited, but AO4B08 was not. Staining intensity: +, comparable intensity to non-pre-incubated cryosections; +/-, reduced intensity compared to non-pre-incubated cryosections ; -, completely absent staining.

LEGENDS TO FIGURES

Figure 1. Staining of diaphragm cryosections from patients with and without COPD with different anti-glycosaminoglycan antibodies. Cryosections were incubated with antibodies HS4C3 (a), AO4B08 (b), IO3H10 (c), IO3H12 (d), IO4C2 (e), and LKN1 (f). Bound antibodies were visualized using anti-VSV antibody P5D4, followed by a Alexa-488 conjugated Goat anti-Mouse antibody. The staining intensity of the epitope recognized by HS4C3 is decreased in patients with COPD (a2), whereas the staining intensity of the epitope recognized by IO4C2 is increased in these patients (e2). The staining intensity of epitopes recognized by antibodies AO4B08, IO3H10, IO3H12, and LKN1 is similar in patients with and without COPD. Scale bar, 25 μm.

FIGURE 1

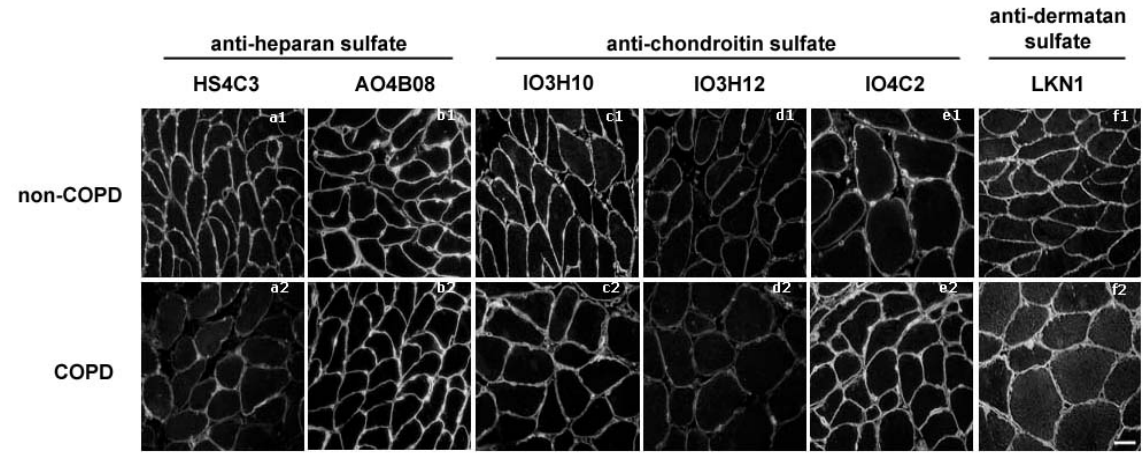


Figure 2. Staining of Heparinase-I/ -III and Chondroitinase-AC treated rat soleus cryosections. Non-treated (a1-c1), Heparinase-I/ -III treated (a2-c2), and Chondroitinase-AC treated (a3-c3) cryosections were incubated without primary antibody (c1), with anti-heparan sulfate antibody HS4C3 (a1-3), anti-chondroitin sulfate antibody IO4C12 (b1-b3), anti-heparan sulfate stub (3G10; c2), or anti-chondroitin sulfate stub (2B6; c3). Bound antibodies were visualized using a rabbit anti-VSV antibody, followed by Alexa-488 conjugated Goat

anti-Rabbit (a1-3; b1-3; c1), or using Alexa-488 conjugated Goat anti-Mouse antibody (c2, c3; control experiment omitting the primary antibody was blank (not shown)). Anti-glycosaminoglycan antibodies stain the muscle endo- and perimysium and capillary endothelium (a1, b1) and this staining disappears upon enzymatic depolymerization of the GAG chains iun question (a2, b3), not when the reciprocal GAG is digested (a3, b2). Staining of heparan sulfate and chondroitin sulfate-stubs confirms the depolymerization of these glycosaminoglycans (c2, c3). Scale bar, 50 μm.

FIGURE 2

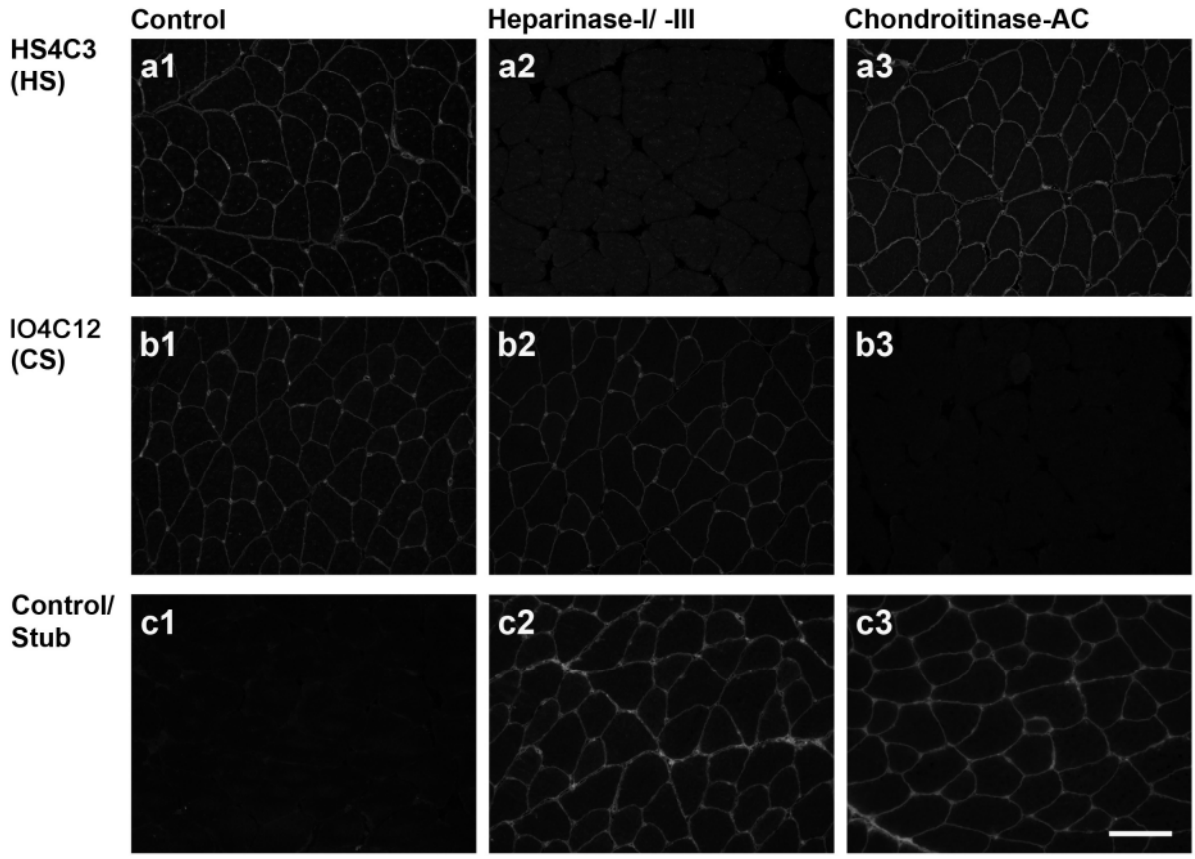


Figure 3. Staining of diaphragm cryosections from patients with (b) and without (a) COPD for proteoglycan core proteins. Cryosections were incubated with antibodies angainst decorin (a1, b1) or perlecan (a2, b2). Bound antibodies were visualized using an Alexa-488

conjugated Goat anti-Mouse antibody. As a control, primary antibodies were omitted (a3, b3). The staining intensity of both decorin (a chondroitin sulfate/ dermatan sulfate proteoglycan present throughout the extracellular matrix) and perlecan (an extracellular matrix heparan sulfate proteoglycan present proximal to the sarcolemma) are similar in patients with and without COPD. Scale bar, 50 μm.

Figure 3

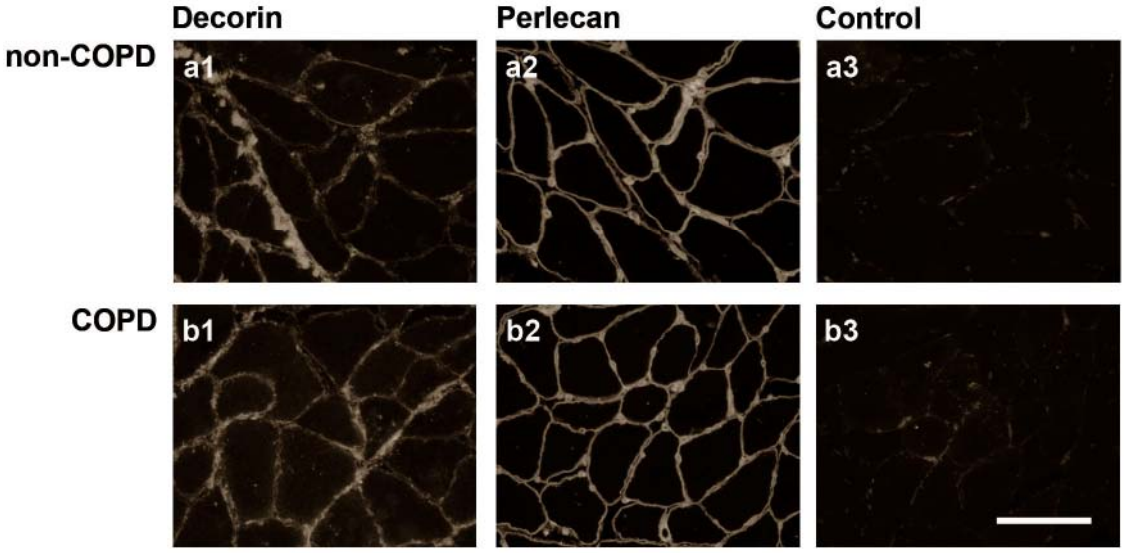


Figure 4. A: Localization of endogenous IGF-I and HGF in diaphragm from non-COPD and COPD patients. Diaphragm cryosections were incubated with anti-growth factor antibodies. Bound antibodies were visualized using anti-VSV antibody P5D4, followed by a Alexa-488 conjugated Goat anti-Mouse antibody. Endogenous IGF-I shows strong cytoplasmic staining of type-I fibers in non-COPD patients (b1), but only little staining in patients with COPD (b2). Compared to non-COPD patients (c1), patients with COPD show an increased staining of endogenous HGF in the endomysium (c2). Scale bar: 50 μm.

B: Double-staining of non-COPD diaphragm with anti-IGF-1 and with anti-myosin heavy chain type I or type II. IGF-1 staining in diaphragm fibers was present predominantly in type I fibers.

FIGURE 4A

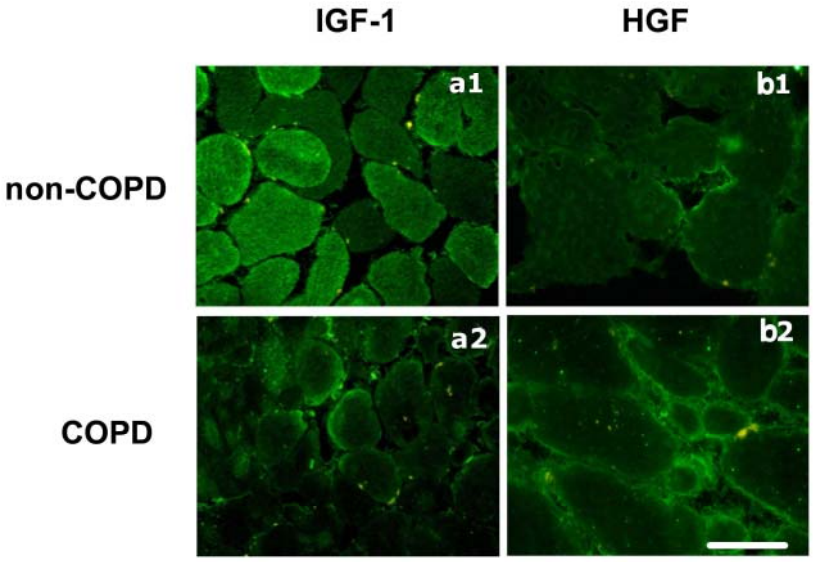


FIGURE 4B

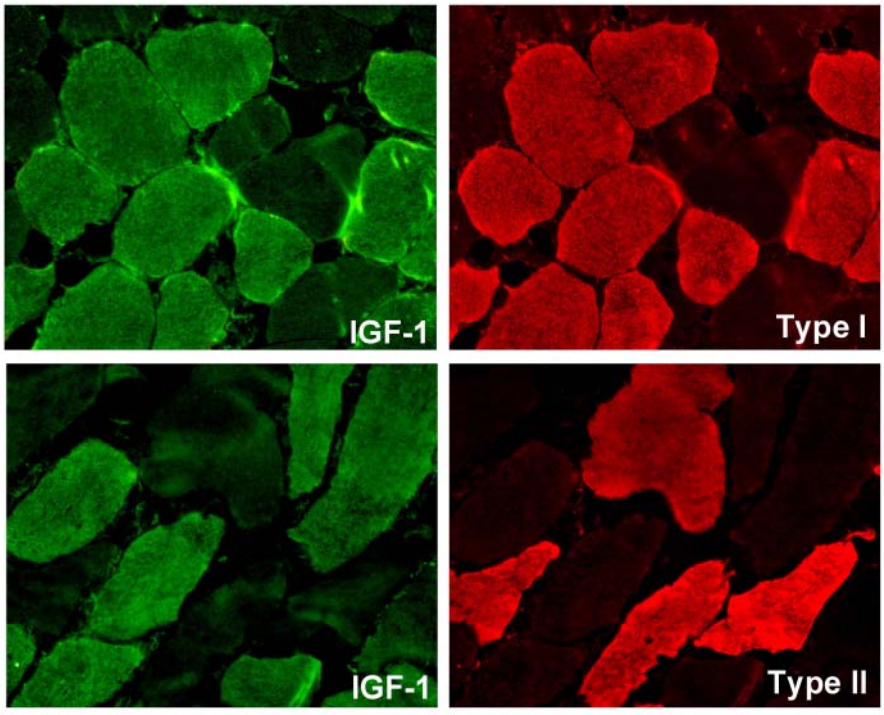
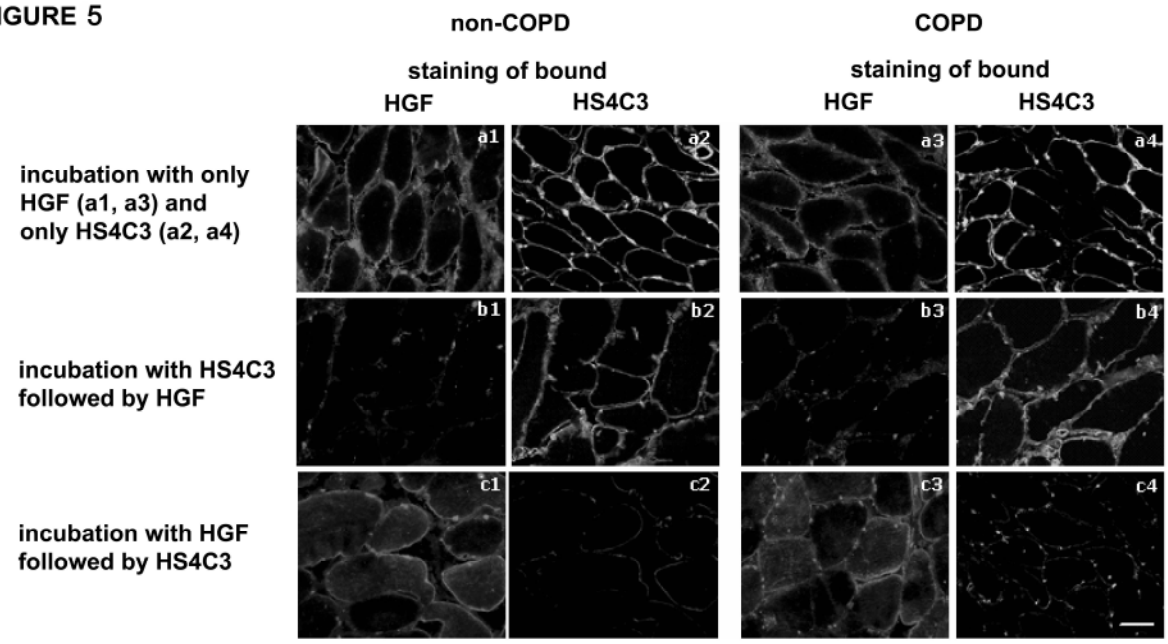


Figure 5. Interference of heparan sulfate binding by HGF and HS4C3. Incubation with only HGF shows strong staining of the endomysium in diaphragm cryosections from non-COPD (*column 1-2*) and COPD patients (*column 3-4*) (*a1 and a3*). Incubation with only HS4C3

shows strong staining of the endomysium (*a2 and a4*). Cryosections from non-COPD and COPD patients were either pre-incubated with HS4C3 (*b*) followed by incubation with HGF, or pre-incubated with HGF (*c*) followed by incubation with HS4C3. Bound HS4C3 and HGF were visualized by double-staining. Following pre-incubation with HS4C3 (*b2 and b4*) cryosections show no staining of HGF (*b1 and b3*). Similarly, when pre-incubated with HGF (*c1 and c3*) cryosections show no staining of HS4C3 (*c2 and c4*). Scale bar: 25 μm.

FIGURE 5



TABLES

Table 1. Patient characteristics

	Non-COPD	COPD
Male/female	5 / 0	8 / 1
Age, yr	58 ± 4	60 ± 3
BMI, $kg*m^{-2}$	28 ± 2	25 ± 2
FEV_1, % predicted	94 ± 7	75 ± 6
VC, % predicted	93 ± 6	97 ± 8
FEV_1/VC, %	77 ± 2	59 ± 2
TLC, % predicted	93 ± 3	103 ± 8
D_{Lco}/VA, % predicted	99 ± 10	82 ± 6
Pao_2, kPa	11.4 ± 0.5	11.6 ± 0.3
$Paco_2$, kPa	5.4 ± 0.4	5.0 ± 0.2

Table 2. Characteristics of the glycosaminoglycan domain-specific antibodies used in this study.

Clone	CDR3	V_H	DP	GAG	Ref.
AO4B08	SLRMNGWRAHQ	3	47	HS[1]	43
HS4C3	GRRLKD	3	38	HS[2]	25
IO3H10	AKRLDW	1	7	CS[3]	44
IO3H12	MKTRLDV	3	46	CS	44
IO4C2	GKQRYS	3	54	CS/ Hep	44
LKN1	GIKL	1	25	DS[4]	45

[1] Recognizes highly sulfated HS epitopes (preferred modifications: IdoA, NS, 2OS, 6OS; preferred domain: [$IdoA_{2S}$-$Glc_{NS,6S}$])
[2] Recognizes highly sulfated HS epitopes (preferred modifications: NS, 3OS, 6OS; preferred domain: [$IdoA_{2S}$-$Glc_{NS,3S,6S}$])
[3] Recognizes CS-C epitopes (preferred domain: [GlcA-$Gal_{Nac,6S}$])
[4] Recognizes DS epitopes (preferred modifications: 2OS, 4OS, 6OS)

Table 3. Immunostaining intensity of anti-glycosaminoglycan antibodies.

	HS4C3	AO4B08	IO3H10	IO3H12	IO4C2	LKN1
Non-COPD						
P1	++	++	++	+	+	+
P2	++	+	++	+/-	-/+	++
P3	++	++	++/+	+/-	+/-	++
P4	++	++	++	+	++/+	++
P5	++	++	++	+/-	+/-	+
COPD						
P6	+	++	++	+	++	+
P7	+/-	++	++	+/-	++/+	++
P8	++/-	++	++	+/-	++	++
P9	+/-	+	++	+	++/-	+
E10	++/+	++	++	+	+	++
P11	-/+	++	++	+/-	++/+	+
P12	+/-	++	+	+	++/+	++
P13	+/-	++/+	++	+	+	++/-
P14	+/-	+	++	+/-	+	+

Table 4.

A. Inhibition of growth factor binding by pre-incubation with anti-heparan sulfate antibodies HS4C3 or AO4B08

	Pre-incubation with HS4C3		Pre-incubation with AO4B08	
	IGF-1	**HGF**	**IGF-1**	**HGF**
Non-COPD				
P1	+	-	+	+
P2	+	-	+	+
P3	+	-	+	+
P4	+	-	+	+
P5	+	-	+	+
COPD				
P6	+	-	+	+
P7	+	-	+	+
P8	+	-	+	+
P9	+	-	+	+
P10	+	-	+	+
P11	+	-	+	-
P12	+	-	+	+
P13	+	+	+	-
P14	+	-	+	-

B. Inhibition of anti-heparan sulfate antibody binding by pre-incubation with growth factors

	Pre-incubation with			
	IGF-1		**HGF**	
	HS4C3	**AO4B08**	**HS4C3**	**AO4B08**
Non-COPD				
P1	+/-	+	+/-	+
P2	+/-	+	-	+
P3	+/-	+	-	+
P4	+/-	+	+/-	+
P5	+/-	+	-	+
COPD				
P6	+/-	+	+/-	+
P7	+/-	+	-	+
P8	+/-	+	+/-	+
P9	+/-	+	-	+
P10	+/-	+	-	+
P11	+	+	-	+
P12	+	+	-	+
P13	+	+	-	+
P14	+	+	-	+