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ARTICLE

Expression of sialyltransferases from the *St3gal*, *St6gal* and *St6galnac* families in mouse skeletal muscle and mouse C2C12 myotubes

Rositsa S. Milcheva*, Any K. Georgieva, Katerina S. Todorova, Svetlozara L. Petkova

Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria

ABSTRACT In skeletal muscles, the sialic acids have a great significance for their functional maintenance and proper structural organization. Our work described the expressions of *St3gal*, *St6gal* and *St6galnac* sialyltransferases specific for glycoproteins in mouse skeletal muscles and murine C2C12 myotubes. Lectin histochemistry, cytochemistry and lectin blot were used to demonstrate the membrane localization and the electrophoretic profiles of α-2,3- and α-2,6-sialylated glycoproteins. The expression levels of sialyltransferases were analysed by real time RT-PCR and western blot. The enzymes *St6gal2* and *St6galnac1* were not expressed in skeletal muscle tissue and C2C12 myotubes. In both experimental groups, mRNAs of the *St3gal* family prevailed over the mRNA expressions of the *St6gal* and *St6galnac* families. The profiles of sialyltransferase expressions showed differences between the two experimental groups, illustrated by the absence of expressions of the mRNA for the *St3gal6* and *St6galnac3* genes in the C2C12 cell samples and by the different shares of the enzymes *St3gal3* and *St3gal4* in both experimental groups. The different patterns of enzyme expressions in both experimental groups corresponded with differences between their α-2,3- and α-2,6-sialylated glycoprotein profiles. These results could be a useful addendum to the knowledge concerning the glycosylation of the skeletal muscle tissue.

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***Corresponding author**
E-mail: rosicamilcheva@abv.bg

Introduction

The attachment of monosaccharide residues is one of the most complicated co- or post translational modifications that proteins can undergo, resulting in an abundant, diverse, and highly regulated repertoire of cellular glycans. Two major classes of oligosaccharides are defined according to the nature of the linkage between the carbohydrate chain and the polypeptide region. An O-glycan (O-linked oligosaccharide) is usually bound to the polypeptide via N-acetylgalactosamine (GalNAc) to a serine (Ser) or threonine (Thr) residue and can be extended into a variety of different structural core classes. An N-glycan (N-linked oligosaccharide) is a sugar chain covalently linked to an asparagine (Asn) residue of a polypeptide chain within the consensus peptide sequence: Asn-X-Ser/Thr (Brockhausen and Stanley 2017; Stanley et al. 2017).

One of the most fascinating building units of the oligosaccharide constructions are the sialic acids. They represent a family of over 40 modifications of the N-acetylneuraminic acid (Neu5Ac). The sialic acids typically occupy the terminal position of the glycoconjugate sugar chains, usually via α-2,3-, α-2,6-, or α -2,8- glycosidic bond (Varki 1992; Harduin-Lepers et al. 2001; Schauer 2004). The glycosidic bonds are generated by highly specific enzymes that belong to four sialyltransferase families. The members of the families, beta-galactoside alpha-2,3-sialyltransferase (ST3Gal), beta-galactoside alpha-2,6-sialyltransferase (ST6Gal) and N-acetylgalactosaminide alpha-2,6-sialyltransferase (ST6GalNAc) are widely spread in different tissues, while the enzymes from the alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase family (ST8SiA) are mostly expressed in the brain (Harduin-Lepers et al. 2005). The sialylation of glycoproteins or glycolipids always occurs into the Golgi, and afterwards they are transported to the cell membrane. Because of their terminal position on the oligosaccharide chains, the sialic acids participate in almost all types of recognition phenomena and adhesion mechanisms (Varki 2007; Schauer 2009).

In skeletal muscles the sialic acids are very important for the functional maintenance of glycoproteins involved in fibre structure and neuromuscular junctions, develop-

ment and regeneration, muscle excitability and exercise performance (McDearmon et al. 2003; Combs and Ervasti 2005; Broccolini et al. 2008; Johnson et al. 2004; Schwetz et al. 2011; Hanish et al. 2013).

Even if the sialylation is not as much abundant as in other tissues, the muscles are very sensitive to sialic acid deficiency due to mutations, which results in a variety of diseases with a severe and progressive loss of motility as a common feature (Tajima et al. 2005; Broccolini et al. 2009). Histological expressions of sialylated glycoproteins in adult human skeletal muscles were already described in detail (Marini et al. 2014). By now, however, the only identified sialylated glycoprotein in skeletal muscles is the α-dystroglycan, a member of the dystrophin-associated glycoprotein complex (Barresi and Campbell 2006). Comprehensive information about the expression of enzymes from the sialyltransferase families is also missing in the available literature.

The aim of this work was to investigate the sialylation in mouse skeletal muscle tissue and C2C12 mouse myotubes in the aspect of localization of α-2,3- and α-2,6-sialylated glycoproteins, relative quantification of sialyltransferase expressions and comparison of the α-2,3- and α-2,6-sialylated glycoprotein profiles.

Material and methods

Ethical procedures

All animal experiments were performed in compliance of Regulation No 20/01.11.2012 on the minimum requirements for protection and welfare of experimental animals and the requirements for the sites for their use, breeding and/or delivery, issued by the Ministry of Agriculture and Food of Republic of Bulgaria.

Mouse tissue samples collection

Five male white laboratory mice, 6-8 weeks old, were humanely euthanized. Tissue specimens were excised from the femoral and gluteal muscles and fixed with freshly prepared modified methacarn fixative (Cox et al. 2006) or stored at -80 °C for further studies. Specimens from lungs, spleen, brain, liver, intestine, colon, and kidneys were archived in low temperatures, too. After processing, the fixed specimens were embedded in paraffin.

Cell cultures

C2C12 mouse myoblast cell line (ATCC® CRL-1772™) was purchased from LGC Standards USA (Manchester, NH, USA). The cells were cultured for 48 h in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) with a high glucose content of 4.0 g/L supplemented with 10% fetal bovine serum (FBS, Gibco-Thermo Fisher Scientific, Waltham, MA, USA), penicillin 100 IU/ml and streptomycin 100 μg/ml, (AppliChem, Darmstadt, Germany) into plastic tissue culture flasks (Orange Scientific, Braine-l`Alleu, Belgium) or onto 12 mm oval glass cover slips (Glaswarenfabrik Karl Hecht, Sondheim, Germany), until 90% confluence of the monolayer was achieved. Further differentiation into myotubes was induced by changing the growth medium to differentiation medium – Dulbecco's Modified Eagle's Medium, supplemented with 2% horse serum (Sigma-Aldrich, Merck, Darmstadt, Germany). After a routine estimation of the fusion index, the mature myotubes were dissociated by 0.05% solution of trypsin (Gibco, Thermo Fisher Scientific) with 0.025% ethylenediaminetetraacetic acid (AppliChem, Darmstadt, Germany) and counted with an automatic cell counter (Countess™, Invitrogen™, Thermo Fisher Scientific). Samples with approximate concentration of 5 x 10^6 cells/ml were stored at -80 °C for further molecular and proteomic studies. The myotube layers onto the cover slips (Glaswarenfabrik Karl Hecht, Sondheim, Germany) were submitted for lectin cytochemistry.

Lectin histo- and cytochemistry

Cover slips with myotube cultures and skeletal muscle tissue sections were treated with biotinylated lectins – *Maackia amurensis* lectin-II (MAL-II, Vector Laboratories, Burlingame, CA, USA), specific for α-2,3-sialic acids (Knibbs et al. 1991) and *Sambucus nigra* agglutinin (SNA, Vector Laboratories), specific for α-2,6-bound sialic acids (Kaku et al 2007). The tissue sections were first rehydrated, and the myotubes were treated with 0.3% Triton in buffer. The further steps were performed in dark. All samples were incubated for 30 min with 1 μg/mL methanol solution of 4′,6-diamidino-2-phenylindole (DAPI, AppliChem), then with SNA or MAL-II (1 μg/mL) for 60 min, and finally with Streptavidin-FITC (1:100, Sigma-Aldrich) for 30 min. Control samples were treated with buffer instead of lectins. The samples were mounted in Vectashield mounting medium (Vector Laboratories) and observed with light microscope Leica DM 5000B (Leica Camera AG, Wetzlar, Germany) under UV, blue, green and UV/violet fluorescent filters. The obtained parallel images of each sample were merged using ImageJ (version 1.48h3; developed at the U.S. National Institutes of Health (Bethesda, MD, USA) by W. Rasband, and available at https://imagej.net/Downloads; Schneider et al., 2012).

Gene expression analyses

The experiments described in this section were designed to evaluate the expression of mRNA of mouse sialyltransferases *St3gal1, 2, 3, 4* and *6*, *St6gal1* and *2*, and *St6galnac1, 2, 3* and *4* in mouse skeletal muscle tissue samples and mouse C2C12 myotubes. The levels of expressions were

estimated via normalization versus the expressions of peptidyl prolyl isomerase A (*Ppia*) and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) as reference genes. All primers were designed using the NCBI Blast Tool (Ye et al. 2012) in a way to span at least one intron sequence. The full names of investigated genes, the primer sequences and the size of the amplified products are available in Table 1, as a supplementary file. The oligonucleotides were purchased from HVD Biotech Vertriebs (Vienna, Austria). The substrate specificities of all sialyltransferases, analyzed in this study, are shown in Table 2.

Five skeletal muscle tissue samples with approximate weight of 30 mg each and five aliquots of C2C12 cell cultures from different passages with approximate concentration of 5 x 10^6 cells/ml were homogenized using TissueRuptur II homogenizer (Qiagen, Hilden, Germany) on ice. Total RNA was isolated and purified by GeneMatrix Universal RNA Purification Kit (EurX®, Gdansk, Poland), strictly following the corresponding protocols recommended by the producer. The yield and purity of the collected RNA were measured using S-300 spectrophotometer (Boeco, Hamburg, Germany).

Approximately 2 μg total RNA from each sample were used for first strand cDNA synthesis. The reverse transcription reaction mixture contained 5 x reaction buffer, 20 U RiboLock Rnase Inhibitor, 1 mM dNTPs, 100 pmol random hexamer primers, 200 U RevertAid Reverse Transcriptase (all of them Fermentas; Thermo Fisher Scientific, Waltham, MA, USA) and diethylpyrocarbonate (DEPC)-treated water (Sigma-Aldrich). The reaction mixture was first incubated at room temperature for 10 min, then at 42 °C for 1 h, and the reaction was terminated at 70 °C for 10 min. The generated cDNA was quantified, and the samples were stored at -80 °C.

Table 1. The full names of the investigated genes and their primers sequences used in this study.

Gene	Abbreviation	Accession number	Primers sequences (5`-3`)	Product size (bp)
Peptidylprolyl isomerase A	*Ppia*	NM_008907.1	TTCGAGCTCTGAGCACTGG – F CCAGTGCCATTATGGCGT – R	115
Glyceraldehyde 3-phosphate dehydrogenase	*Gapdh*	NM_001289726 transcript variant 1	TCCTCGTCCCGTAGACAAAATG – F AATCTCCACTTTGCCACTGC – R	103
ST3 beta-galactoside alpha-2,3-sialyltransferase 1	*St3gal1*	NM_009177.4	ACCATCACTCACACCTATGTCC – F CCTGAAGCCAGTTGTCAAAGAC – R	112
ST3 beta-galactoside alpha-2,3-sialyltransferase 2	*St3gal2*	NM_009179	TCCTTCCTTCGAGTGGACAAAG – F ACCAGCATTCCTGTGGAAGG – R	116
ST3 beta-galactoside alpha-2,3-sialyltransferase 3	*St3gal3*	NM_001161774.2 transcript variant 2	AACTTTTCCGAGGGAGCTTG – F TAGCCCACTTGCGAAAGGAG – R	118
ST3 beta-galactoside alpha-2,3-sialyltransferase 4	*St3gal4*	NM_009178.4	TGGGTAAAGACGCCATCCAC – F TCGAGGCTCTTTATGCTCTCAG – R	119
ST3 beta-galactoside alpha-2,3-sialyltransferase 6	*St3gal6*	NM_018784.2	TCCCAGCTGAAGAAATGAGGAC – F TCAGCTCTGCACAGAAATGG – R	112
ST6 beta-galactoside alpha-2,6-sialyltransferase 1	*St6gal1*	NM_145933.3	GCCGTCGTGTCTTCTGCAGGAT – F TGGAAGTTGTCTGTAGGTGCCCC – R	107
ST6 beta-galactoside alpha-2,6-sialyltransferase 2	*St6gal2*	NM_172829.2	CTGCGCAGTTGTCATGTCTG – F TTTCTCATAGCCACGTGTAGGG – R	115
ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)1-acetogalactosaminide alpha-2,6-sialyltransferase 1	*St6galnac1*	NM_011371.2	TCCTGCTTCTGACTGTGTTGGCA – F TCTCCTGGGCACTTGCGTCA –R	117
ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)1-acetogalactosaminide alpha-2,6-sialyltransferase 2	*St6galnac2*	NM_009180.3	CCCACGAGCATTCTTTGACCCCA – F TCAAACAGGCTGCGGAAGCGA – R	117
ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)1-acetogalactosaminide alpha-2,6-sialyltransferase 3	*St6galnac3*	NM_011372.2	CAGGCAGCCTCTTCGAACTCACT – F ACCTTCTGCCCGACCATTTGACC – R	117
ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)1-acetogalactosaminide alpha-2,6-sialyltransferase 4	*St6galnac4*	NM_011373.3 transcript variant 1	TTCACTGAACGCATGATGGC – F AGGGCCAGAATCATGGTGAAC – R	116

Real-time PCR was designed on approximately 700 ng cDNA as a template in 20 µl total volume of reaction using SG qPCR Master Mix (2x), 0.25 U uracyl-N-glycolase (UNG), nuclease free water (all from EurX) and 0.2 µM R- and F-primers specific for amplification of fragments of *Gapdh*, *Ppia*, *St3gal1*, *2*, *3*, *4* and *6*, *St6gal 1* and *2*, and *St6galnac1*, *2*, *3* and *4*. Three real-time PCR reactions/sample in duplicate were performed for amplification of each fragment of interest using RotorGene™ 6000 Real-time Analyzer (Corbett Life Science-Qiagen).

The data were analysed using Rotor Gene Q Series Software (Qiagen) and the relative quantification of the sialyltransferase expressions was calculated by the ΔΔCt method (Zhang et al. 2014) versus *Ppia* and *Gapdh* as reference genes. After each run, a High-Resolution Melting Curve Analysis (HRM) was performed to verify the specificity of the amplified products, which were visualized on 2.5% agarose gel supplemented with Simply Safe nucleic acid stain (EurX) versus 100-1000 bp DNA Ladder (EurX) and the gels were photographed with a gel documentation system Vision (Scie-Plas Ltd, Cambridge, UK).

Statistical analysis of the gene expression quantification

Statistical analysis of the data was performed using GraphPad Prism 5.03 software (San Diego, CA, USA). One Way Anova analysis with test of Bonferroni was computed to detect statistically significant differences between the Ct values of the qPCR products, and the results were interpreted as follows: $P < 0.001$ = highly significant; $P < 0.01$ = very significant; $P < 0.05$ = significant.

SDS-PAGE, lectin- and western blotting

Skeletal muscle tissue samples with an approximate weight of 30 mg each and aliquots of C2C12 myotubes from different passages with an approximate concentration of 5 x 10^6 cells/ml were homogenized in 0.6 M Tris buffer, containing 150 mM NaCl, 5 mM EDTA and 1% CHAPS (all purchased from Sigma-Aldrich), supplemented with Proteinase inhibitor cocktail, set 3 (Sigma-Aldrich), using TissueRuptur II homogenizer (Qiagen) on ice, and then centrifuged at 21 000 g, for 1 h at 4 °C. The supernatants were used for methanol/chloroform protein precipitation, as described by Fic et al. (2010). The protein pellet was reconstituted in 6 M urea buffer, containing 1.5 M thiourea, 3% CHAPS, and 66 mM DTT (all purchased from Sigma-Aldrich), and stored at -20 °C. The protein content was measured by the method of Bradford (1976) on spectrophotometer S-300 (Boeco).

Approximately 30 µg from each sample were mixed with 4 x Loading buffer (EurX), samples were heated at 98 °C for 10 min and were then loaded on 10% polyacrylamide gel. SDS-PAGE was performed under reducing conditions as described by Laemmli (1970).

Gels were then stained with colloidal Coomassie Brilliant Blue (Jahn et al. 2013) or were forwarded to western blotting on 0.45 µm nitrocellulose membranes (Sigma-Aldrich), as described by Towbin et al. (1979). The membranes designated for lectin-affino blots were blocked with 5% non-fat dry milk (Sigma-Aldrich) for 1 h, then

Table 2. Substrate specificity of the sialyltransferases, operating on glycoproteins (Markos et al. 2004; Takashima 2008), investigated in this study. The monosaccharides in bold indicate a residue onto which a sialic acid is transferred via α-2,3- or α-2,6-glycosidic linkage. Gal – galactose, GalNAc – N-acetyl-D-galactosamine, GlcNAc – N-acetyl-D-glucosamine, SiA – sialic acid, Ser – serine, Thr – threonine.

β-Galactoside-α-2,3-sialyltransferase family (ST3Gal)	
ST3Gal1	**Gal**-β-1,3-GalNAc
ST3Gal2	**Gal**-β-1,3-GalNAc
ST3Gal3	**Gal**-β-1,3-GlcNAc > **Gal**- β-1,4-GlcNAc > **Gal**-β-1,3-GalNAc
ST3Gal4	**Gal**-β-1,3-GalNAc > **Gal**- β-1,4-GlcNAc > **Gal**-β-1,3-GlcNAc
ST3Gal6	**Gal**- β-1,4-GlcNAc > **Gal**-β-1,3-GlcNAc
β-Galactoside-α-2,6-sialyltransferase family (ST6Gal)	
ST6Gal1	**Gal**- β-1,4-GlcNAc
ST6Gal2	**Gal**- β-1,4-GlcNAc
GalNAc α-2,6-sialyltransferase family (ST6GalNAc)	
ST6GalNAc1	**GalNAc**-α-1-Ser/Thr (Tn Ag) > Gal-β-1,3-**GalNAc**-α-1-Ser/Thr (T Ag)
	SiA-α-2,3-Gal-β-1,3-**GalNAc**-α-1-Ser/Thr (sialyl-T Ag)
ST6GalNAc2	Gal-β-1,3-**GalNAc**-α-1-Ser/Thr > **GalNAc**-α-1-Ser/Thr
	SiA-α-2,3-Gal-β-1,3-**GalNAc**-α-1-Ser/Thr
ST6GalNAc3	SiA-α-2,3-Gal-β-1,3-**GalNAc**-
ST6GalNAc4	SiA-α-2,3-Gal-β-1,3-**GalNAc**-

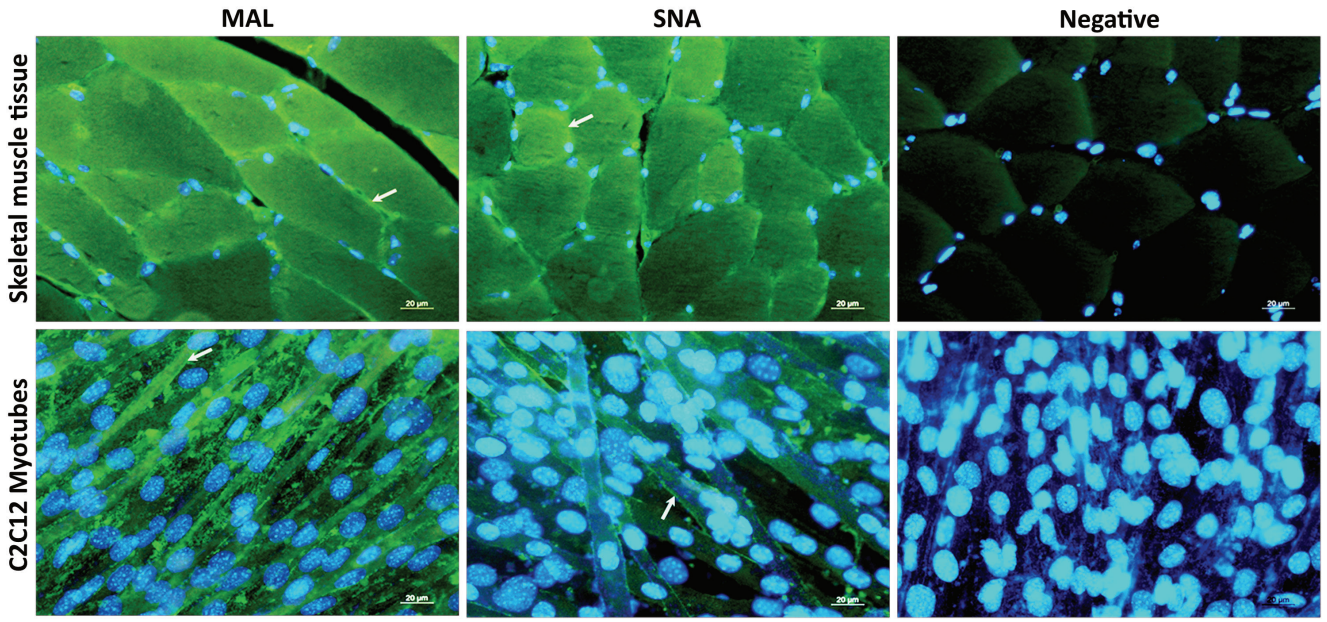

Figure 1. Sialylated glycorpoteins in mouse skeletal muscle tissue and mouse C2C12 myotubes. Skeletal muscle tissue sections and C2C12 myocyte cultures were stained with lectins MAL-II and SNA specifically recognizing α-2,3- and α-2,6-sialylated glycoproteins located on the cell membranes (arrows). Streptavidin-FITC, DAPI. Scale bar: 20 µm.

incubated with biotinylated SNA (Vector Laboratories) or MAL-II (Vector Laboratories) (1 µg/mL) for 1 h, and finally treated with streptavidin horseradish peroxidase (HRP, Vector Laboratories) for 30 min at room temperature. The membranes designated for western blots were treated with goat blocking serum (Vector Laboratories) for 1 h, then incubated with polyclonal rabbit antibodies against GAPDH (1:2000, Thermo Fisher Scientific), ST3Gal VI (1 µl/mL) and ST6GalNAc III (2 µl/mL) (Sigma-Aldrich) for 2 h, and finally treated with WestVision Peroxidase Polymer Anti-Rabbit IgG (Vector Laboratories) for 30 min at room temperature. The colour reaction on all membranes was developed after exposure to DAB Peroxidase Substrate solution (Vector Laboratories). The approximate molecular weight of the detected protein bands was estimated versus Perfect™ Tricolor Protein Ladder (EurX), ranging from 11 to 245 kDa.

Results

Localization and protein profiles of α-2,3- and α-2,6-sialylated glycoproteins

The lectin histochemistry and cytochemistry in our study demonstrated the membrane localization of α-2,3- and α-2,6-sialylated glycoproteins in mouse skeletal muscle samples and the C2C12 cell line (Fig. 1). The cell line samples were much more reactive towards MAL-II in comparison with SNA. Both experimental groups showed similar protein profiles with a slight difference between the patterns of the α-2,3-sialylated glycoproteins, demonstrated by MAL-II affino-blot. The C2C12 cell culture samples showed a higher number of α-2,6-sialylated glycoproteins, as demonstrated by the SNA affino-blot. In both experimental groups, the lectin affino-blots revealed

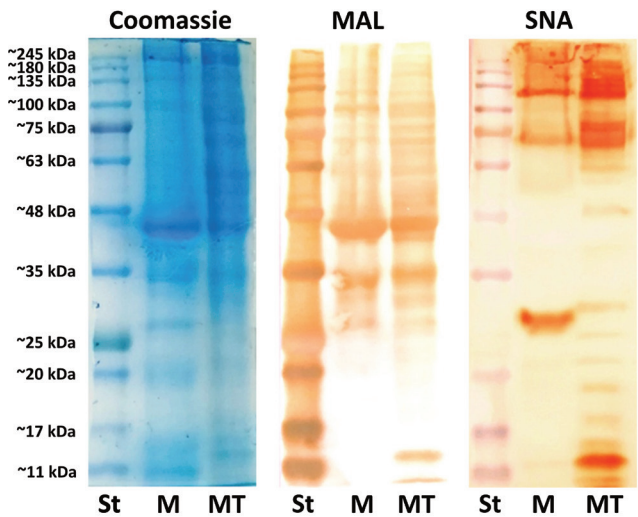

Figure 2. SDS-PAGE, MAL-II and SNA lectin affino-blot of mouse skeletal muscle tissue (M) and C2C12 myotube samples (MT), loaded on 10% gels versus Perfect™ Tricolor Protein Ladder (EurX) ranging from 11 to 245 kDa (line L), showing different patterns of sialylation. Streptavidin-HRP, DAB.

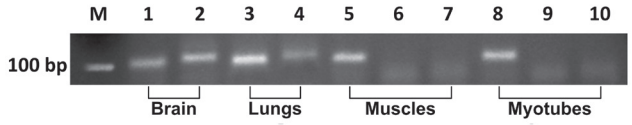

Figure 3. Absence of amplification products specific for mouse *St6gal2* and *St6galnac1* sialyltransferases in mouse skeletal muscles and C2C12 myotubes. Mouse brain and lungs were used as positive expression controls. Lanes: M – 100 bp fragment of DNA Ladder, 1 and 2 – *Gapdh* (103 bp) and *St6gal2* (115 bp) expressions in brain, 3 and 4 – *Gapdh* and *St6galnac1* (117 bp) expressions in mouse lungs, 5, 6 and 7 – *Gapdh*, *St6gal2* and *St6galnac1* expressions in mouse skeletal muscles, 8, 9 and 10 – *Gapdh*, *St6gal2* and *St6galnac1* expressions in C2C12 myotubes.

sialylated glycoproteins with an approximate molecular weight between 120 and 15 kDa (Fig. 2).

Expression of sialyltransferases

Our study was designed to analyse the expressions of members from the β-galactoside α-2,3-sialyltransferase (*St3gal*), β-galactoside α-2,6-sialyltransferase (*St6Gal*) and GalNAc α-2,6-sialyltransferase (*St6galnac*) families, operating on glycoproteins (Takashima 2008), which substrate preferences were described in Table 2.

The mRNAs of *St6gal2* and *St6galnac1* didn't show products of amplification in the skeletal muscle samples and in the C2C12 cell line (Fig. 3). Expressions of mRNAs for all the rest of the sialyltransferases were detected in mouse skeletal muscle samples (Fig. 4A). Expressions of mRNA for the genes *St3gal6* and *St6galnac3* were not detected in the C2C12 muscle cell samples (Fig. 4B) and this was confirmed also by protein western blot (Fig. 4C).

According to the percent distribution analysis of the expressions of investigated sialyltransferases in both experimental groups (Fig. 5), mRNAs of the *St3gal* family prevailed over the mRNA expressions of the *ST6gal* and *St6galnac* families. The profiles of sialyltransferase

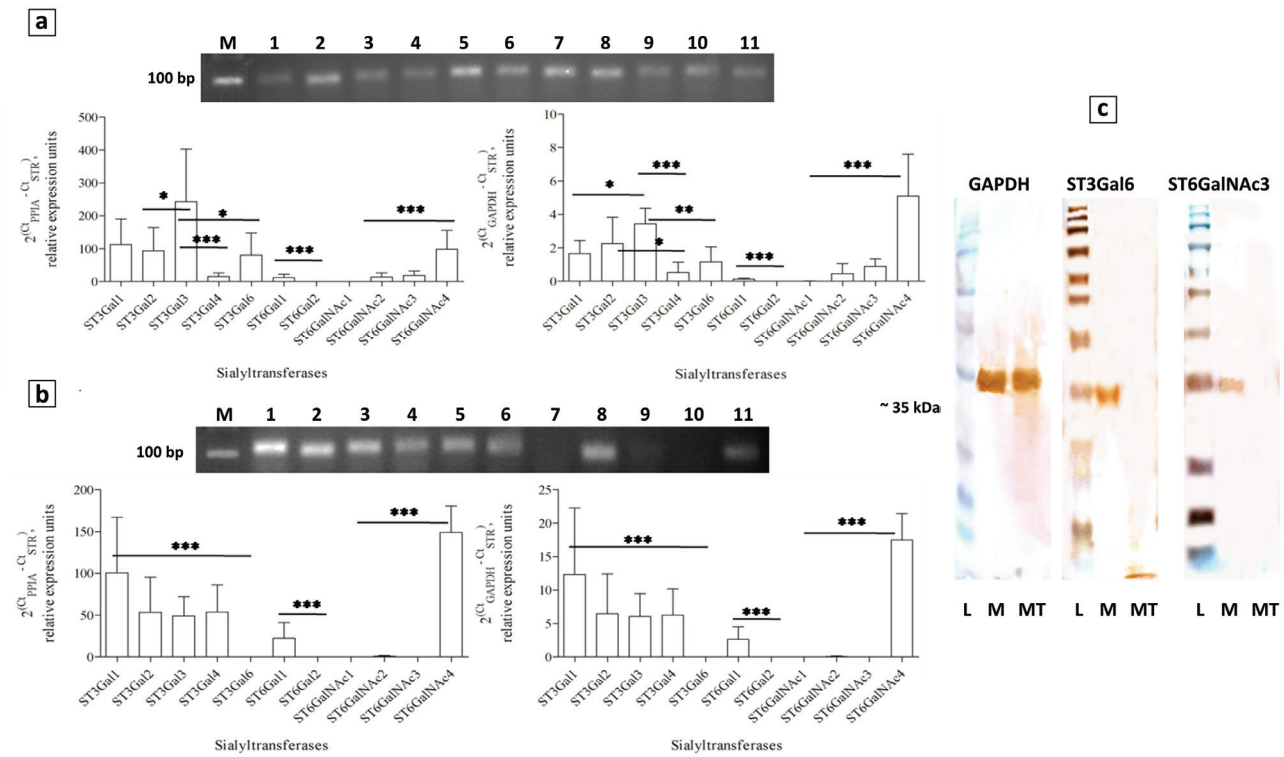

Figure 4. Expressions of mouse sialyltransferases analyzed by real time RT-PCR in skeletal muscle tissue (a) and C2C12 myotube cultures (b), and by western blot (c). Panels a and b – The photographs show amplification products specific for mouse sialyltransferases on 2.5% agarose gel: M – 100 bp fragment of DNA Ladder, 1 – **Ppia** (115 bp), 2 – *Gapdh* (103 bp), 3 – *St3gal1* (112 bp), 4 – *St3gal2* (116 bp), 5 – *St3gal3* (118 bp), 6 – *St3gal4* (119 bp), 7 – *St3gal6* (112 bp), 8 – *St6gal1* (107 bp), 9 – *St6galnac2* (117 bp), 10 – *St6galnac3* (117 bp), 11 – *St6galnac4* (116 bp). The charts represent a relative quantification of sialyltransferase expressions calculated by the ΔΔCt method versus *Ppia* (left) and *Gapdh* (right) as reference genes from six individual samples in triplicate. The bars show the standard deviation. The stars indicate statistically significant difference between sialyltransferase expressions in each family: *** P < 0.001, ** P< 0.01, * P< 0.05. Panel c – Western blots of mouse skeletal muscle tissue (M) and C2C12 myotube samples (MT), with polyclonal rabbit antibodies against GAPDH Thermo Fisher Scientific), ST3Gal VI and ST6GalNAc III (Sigma-Aldrich) sialyltransferases, loaded on 10% gels versus Perfect™ Tricolor Protein Ladder (EurX) ranging from 11 to 245 kDa (line L), showing absence of expression of both enzymes by the C2C12 myotubes. ImPress™ HRP Anti-Rabbit IgG, DAB.

expressions were different between skeletal muscle tissue samples and C2C12 cell cultures, illustrated by the missing expressions of the mRNA for the *St3gal6* and *St6galnac3* genes in the C2C12 cell samples and by the different shares of the genes *Stgal3* and *Stgal4* in both experimental groups. Among the members of the *St6galnac* family, the expression of *St6galnac4* gene prevailed strongly in both experimental groups. The expression of the *St6galnac2* sialyltransferase was also significantly lower in the C2C12 myotubes. Both experimental groups showed expression of only *St6gal1* but not *St6gal2* (data not shown).

Discussion

Apart from the broad knowledge about the extracellular proteoglycan components and their role in the muscle growth and development (Velleman 2002), most of the information concerning glycosylation of the skeletal muscle tissue is related to inherited disease states (Grewal and Hewitt 2003; Martin-Rendon and Blake 2003) and actually very little is known about its normal glycoproteome.

As already mentioned, the only sialylated glycoprotein discovered in the skeletal muscle tissue by now, was the α-dystroglycan bearing α-2,3-linked sialic acid residues (Barresi and Campbell 2006). Our results showed however the presence of at least several α-2,3- and α-2,6-sialylated glycoproteins, still not identified.

A very important aspect in this scientific topic is the expression of sialyltransferases in muscles. The great variety of the oligosaccharide constructions used as acceptors by the sialyltransferases predetermines the diversity of these enzymes, which were grouped into four families according to the glycosidic linkages they synthesize. From amino acid sequence similarities, substrate specificities and gene structures, the members of each sialyltransferase family were classified into subfamilies (Harduin-Lepers et al. 2005).

In mice and humans, ST6Gal II is one of the two members of the ST6Gal family. Both members utilize the Gal-β-1,4-GlcNAc structure on glycoproteins and oligosaccharides as acceptor substrates. The *St6gal1* gene has a wide range of tissue expression; however, the *St6gal2* gene is expressed in a stage-specific (embryonic stage) and a tissue-specific (adult brain) manner (Takashima et al. 2003), as confirmed also by our results.

The enzymes ST6GalNac I and ST6GalNAc II were classified into a common subfamily of the ST6GalNAc family. Both enzymes exhibit similar substrate specificity, utilizing GalNAc- (Tn antigen), Gal-β-1,3-GalNAc- (T antigen) and SiA-α-2,3-Gal-β-1,3-GalNAc- (sialyl-T antigen) structures on O-glycans of glycoproteins as acceptor substrates (Kono et al. 2000). However, ST6GalNAc I

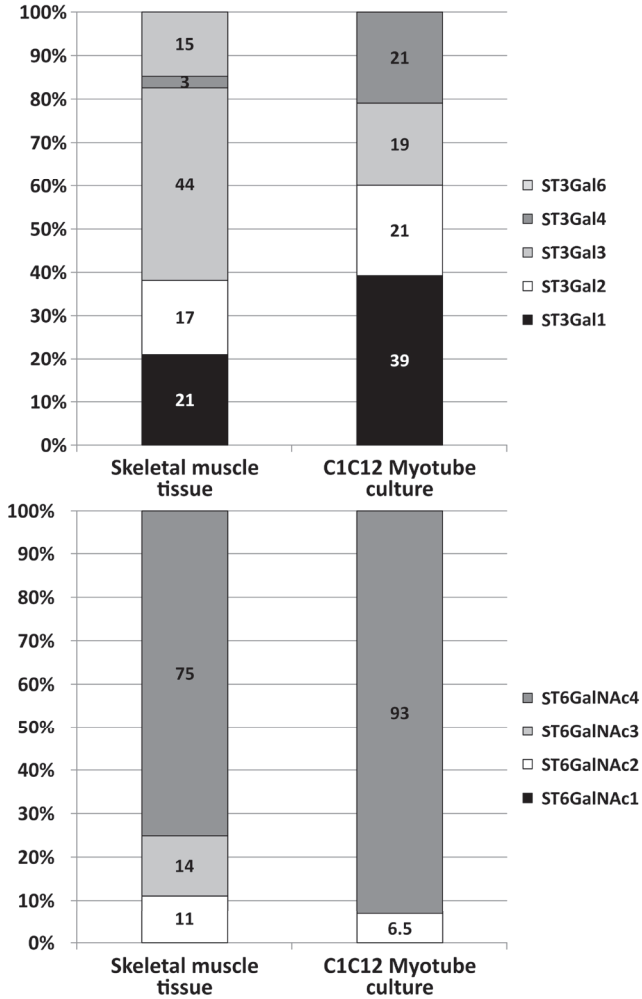

Figure 5. Percent distribution of the gene expressions of the enzymes sialyltransferases from the *St3gal* and *St6galnac* families in mouse skeletal muscle and mouse C2C12 myotubes. Normalization shown versus *Ppia* as a reference gene.

was reported as a major sialyl-Tn synthase, whereas the ST6GalNAc II acts preferentially on T antigen (Markos et al. 2004). In our study, we observed a positive signal of amplification of *St6galnac1* specific product in mouse lung tissue, but not in our experimental muscle and myotube samples.

ST3Gal VI is a member of the ST3Gal family and utilizes preferentially the Gal-β-1,4-GlcNAc structure on glycoproteins and glycolipids as an acceptor substrate (Okajima et al. 1999). ST6GalNAc III together with ST6GalNAc IV was classified into a common subfamily from the corresponding sialyltransferase family. These two enzymes utilize the SiA-α2,3-Gal-β-1,3-GalNAc (sialyl-T antigen) structure on glycoproteins as an acceptor substrate (Lee et al. 1999). Our results definitively showed absence of expression of *St3gal6* and *St6galnac3*

and their encoded proteins in C2C12 myotubes, a finding that is in agreement with similar results predicted by Janot et al. (2009).

Another intriguing difference between the muscle tissue and the cell culture was the different expression levels of the genes *St3gal3* and *St3gal4*, which belong to the same subfamily of α-2,3-sialyltransferases. Both enzymes utilize the same oligosaccharide structures as substrate acceptors, but with quite opposite preferences (Takashima 2008).

The development of new technologies in life sciences opened in the late 1980s a new division of molecular biology named "glycobiology". Since then, huge knowledge was accumulated concerning the chemistry of carbohydrates, the enzymology of glycan biosynthesis and degradation, the structure of glycoconjugates, the recognition of glycans by specific proteins and the roles that the glycans occupy in complex biological systems. In this rapidly growing field in the natural sciences, however, the skeletal muscles remained somehow not quite well explored object of investigation. The results from our work could be a useful addendum to the knowledge concerning the glycosylation of this tissue. In addition, this report would be helpful and informative for any research in future where the C2C12 cell cultures will take a place as an experimental model.

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