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Original article

ABSENCE OF ST3GAL2 AND ST3GAL4 SIALYLTRANSFERASE EXPRESSIONS IN THE NURSE CELL OF *TRICHINELLA SPIRALIS*

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Summary

Milcheva, R. S., P. Janega, S. L. Petkova, K. S. Todorova, D. G. Ivanov & P. Babál, 2022. Absence of ST3Gal2 and ST3Gal4 sialyltransferase expressions in the nurse cell of *Trichinella spiralis*. *Bulg. J. Vet. Med.,* **25**, No 1, 130–138.

This study was aimed to describe some glycosylation changes in the Nurse cell of *Trichinella spiralis* in mouse skeletal muscle. Tissue specimens were subjected to lectin histochemistry with *Maackia amurensis* lectin (MAL), Peanut agglutinin (PNA) and neuraminidase desialylation in order to verify and analyse the structure of α-2,3-sialylated glycoproteins, discovered within the affected sarcoplasm. The expressions of two sialyltransferases were examined by immunohistochemistry. It was found out that the occupied portion of skeletal muscle cell responded with synthesis of presumable sialyl-T-antigen and α-2,3-sialyllactosamine structure, that remained accumulated during the time course of Nurse cell development. The enzymes β-galactoside-α-2,3-sialyltransferases 2 and 4, which could be responsible for the sialylation of each of these structures, were however not present in the invaded muscle portions, although their expressions in the healthy surrounding tissue remained persistent. Our results contribute to the progressive understanding about the amazing abilities of *Trichinella spiralis* to manipulate the genetic programme of its host.

Key words: Nurse cell, sialic acids, sialyltransferases, *Trichinella spiralis*

INTRODUCTION

Trichinellosis is a food borne disease in humans and animals, caused by parasitic nematodes from the *Trichinella* genus. Twelve species have been identified so far, which are classified into two clades. The clade of encapsulated species includes *T. spiralis*, *T. britovi*, *T. nativa*, *T. murrelli*, *T. nelsoni*, *T. patagoniensis*, T6, T8 and T9. The clade of nonencapsulated species includes *T. pseudospiralis*, *T. papuae*, and *T. zimbawensis* (Muñoz-Carrillo *et al.,* 2018). The invasion begins

in the small intestine after consumption of raw or undercooked meat, contaminated by infectious L1 larvae. After four subsequent molts the larvae reach adolescence and reproduce. The chronic stage occurs only in the striated muscles, where the newborn larvae accommodate. After penetrating the skeletal muscle cell, they induce dramatic genetic, morphological and functional changes, resulting in a unique structure called nurse cell, which is capable of supporting the parasite for years (Despommier, 1998; Muñoz-Carrillo *et al.,* 2018).

The nurse cell, mostly of *T. spiralis*, has been a subject of many research studies because it is like nothing else in Nature. This structure derives from a small portion of the occupied sarcoplasm that subsequently undergoes infection-induced cell cycle re-entry and suspension in apparent G2/M phase (Patra & Sarkar, 2014). During the process of de-differentiation, at least 53 genes associated with apoptosis, satellite cell activation and proliferation, cell differentiation, cell proliferation and cycle regulation, myogenesis and muscle development change their expression (Wu *et al.,* 2008).

One of the most complicated co- or posttranslational modifications that proteins can undergo is the attachment of monosaccharide residues. The majority of glycosylated proteins are divided in two main groups, depending on the amino acid onto which the glycosylation occurs. The N-glycans are connected to asparagine residues, and the O-glycans – to serine or threonine residues (Brockhausen & Stanley, 2017; Stanley *et al.,* 2017). The most abundant terminal monosaccharide modifications on glycoconjugates are the sialic acids, which are derivates of the N-acetyl neuraminic acid and are widely distributed in higher animals and some microorganisms (Schauer, 2004). After being transferred by enzymes belonging to different sialyltransferase families, the sialic acids could be attached to the penultimate sugar residue via α-2,3-, α-2,6- or α-2,8-glycosidic bond. Because of their outer position on the oligosaccharide chains, the sialic acids are involved in almost all types of recognition phenomena and adhesion mechanisms, which predetermine their membrane localisation (Varki *et al.,* 2017).

Even if the sialylation in skeletal muscles has not been studied in details, many reports indicated the importance of the sialic acids for the functional maintenance of glycoproteins involved in cell structure and neuromuscular junctions (McDearmon *et al.,* 2003; Combs & Ervasti, 2005), development and regeneration (Broccolini *et al.,* 2008), muscle excitability (Schwetz *et al.,* 2001; Johnson *et al.,* 2004) and exercise performance (Hanish *et al.,* 2013). Previous investigations of ours have shown for the first time that the muscle phase of trichinellosis was associated with glycosylation changes and increased sialic acid synthesis within the occupied areas of the muscle cell (Milcheva *et al.,* 2015; 2019).

The present work describes the intracellular expression of α-2,3-sialylated glycoproteins and their possible association with two α-2,3-sialyltransferases.

MATERIALS AND METHODS

Animals, invasion, sample collection and preparation

Infective *Trichinella spiralis* larvae were isolated from previously invaded mice, between post infection days 30 and 40 according to a routine protocol, as previously described (Milcheva *et al.,* 2019).

White laboratory mice, 6–8 weeks of age, were inoculated with 500 infective *T. spiralis* larvae *per os*. The animals were sacrificed at day 0, 12, 18, and 30 post invasion (d. p. i.) and skeletal muscle specimens (front and hind limbs, pectoral and gluteal muscles) were excised and fixed with 10% neutral buffered formalin (NBF). After processing, the specimens were embedded in paraffin. Sections, 5 μm thick, were deparaffinised in xylene, hydrated in alcohol solutions with decreasing concentrations and water, and then stained with haematoxylin and eosin (H&E) for basic morphological evaluation. All animal experiments were performed in compliance with the Institutional Guidelines for Animal Experiments of IEMPAM-BAS.

Lectin histochemistry

Parallel tissue sections, 5 μm thick, were submitted to histochemistry with biotinylated *Mackia amurensis* lectin (MAL, Vector, Burlingame CA, USA) and Peanut agglutinin (PNA, Sigma-Aldrich, Saint Louis, USA). The carbohydrate specificity of the two lectins used in this study is listed in Table 1. Deparaffinised sections were rehydrated in Tris-buferred solution (TBS) pH 7.4, containing 0.1% Tween 20. Endogenous peroxidase was blocked with En Vison^{TM} Peroxidase Block (Dako, Glostrup, Denmark) and endogenous biotin was blocked with Biotin blocking system (Dako). The sections were incubated with MAL (2 μg/mL) and PNA (10 μg/mL) for 1 hour at room temperature. Part of the sections were incubated with 0.5 U/mL neuraminidase from *Clostridium perfrigens* (Sigma-Aldrich), that specifically cleaves α-2,3-sialic acid residues, in 0.05M sodium acetate buffer (pH 5.5), at 37^{o}C for 15 hours before application of peroxide and biotin blocking systems. To check the binding specificity, the lectins in part of the experiments were pre-incubated with 0.1M solutions of target monosaccharides for 1 hour at room temperature: 3`-sialyl lactose (Calbiochem-Novabiochem, San Diego CA, USA) for MAL and D-galactose for PNA (Sigma-Aldrich). Pre-incubation of PNA was intended only for neuraminidase pre-treated sections. In parallel, negative controls were incubated with TBS instead of lectin. All of the sections were treated then with Streptavidin-HRP (Dako) for 30 min, peroxidase activity was developed with En Vision^{TM} 3,3`-diaminobenzidine (DAB+) substrate-chromogen (Dako), the sections were counterstained with haematoxylin, mounted in acrylic resin and examined under Nikon Eclipse 80í light microscope (Nikon, Kanagawa, Japan).

The intra-cellular staining intensity of the affected skeletal muscle cells was evaluated semi-quantitatively versus the surrounding non-affected sarcoplasm by two independent observers into four arbitrary categories: negative (–), weakly (+), moderately (++) and strongly (+++) positive.

Table 1. The lectins used in this study, their abbreviations, carbohydrate specificity, applied dillutions and related references. SiA –sialic acid, Gal – galactose, GalNAc – N-acetyl-D-galactosamine

Lectin	Abbreviation	Carbohydrate specificity	References
Arachis hypogea agglutinin	PNA	Gal-β-1,3-GalNAc	Lotan *et al.* (1975)
Maackia amurensis lectin-II	MAL-II	SiA-α-2,3-Gal-β-1,4-GlcNAc	Knibbs *et al*. (1991)

Table 2. The substrate specificities of the sialyltransferases investigated in this study and the types of the formed linkages. Gal – galactose, GalNAc – N-acetyl-D-galactosamine, GlcNAc – N-acetyl-D-glucosamine. The monosaccharides in bold indicate a residue onto which a SiA is transferred

Enzyme (short name)	Substrate specificity	Type of created linkage	References
ST3Gal2	Gal-β-1,3-GalNAc-	α-2,3-	Kim *et al.* (1996)
ST3Gal4	Gal-β-1,3-GalNAc- > Gal-β-1,4-GlcNAc->Gal-β-1,3-GlcNAc-	α-2,3-	Kitigawa & Paulson (1994)

Immunohistochemistry

Parallel tissue sections, 5 μm thick, were submitted to an antigen retrieval step with Tris-EDTA retrieval solution (Dako) pH 9.2, at sub-boiling temperature in a pressure cooker for 15 min. The sections then were incubated with rabbit polyclonal Ab against α-2,3-sialyltransferases-II and -IV (ST3Gal2, 1:1000 and ST3Gal4, 1:2000) (Aviva Systems Biology, San Diego, CA, USA) for 4 hours at room temperature.

The substrate specificities of the two sialyltansferases analysed in this study are listed in Table 2. The sections were subsequently incubated for 30 minutes with EnVision™ anti-rabbit or anti-mouse polymer conjugated with horseradish peroxidase (Dako) and the immunoreactivity was visualised with 3,3`-diaminobenzidine (Dako). In parallel, negative control specimens were incubated with Dako Real antibody diluents (Dako) instead of Ab and thereafter with the corresponding peroxidase conjugate. The location of ST3Gal2 and ST3Gal4 expressions were evaluated using a Nikon Eclipse 80í light microscope (Nikon).

RESULTS

Histology of affected skeletal muscle cells de-differentiation after T. spiralis invasion

The affected sites in skeletal muscle specimens from day 12 p.i. were distinguished by the centralisation and hypertrophy of nuclei of the cell. On d.p.i. 18 the affected cytoplasm of the cell was progressively disintegrated and until 30 d.p.i. the de-differentiation of the affected cell into a Nurse cell was complete. During this period an exchange of eosinophilic with basophilic cytoplasm was observed followed by restoration of the eosinophilic cytoplasm. The hypertrophied nuclei were permanent characteristics of the process of de-differentiation and persisted also within the capsulated Nurse cell containing coiled larvae.

Lectin histochemistry

Preincubation of MAL with 3`-sialyllactose and of PNA with D-galactose resulted in a complete block of lectin activity (data not shown).

The sarcoplasm of the healthy skeletal muscle cells did not react with the two lectins used in the study, MAL reacted with the sarcolemma and after neuraminidase treatment the staining was lost. After neuraminidase treatment PNA stained intensely the sarcolemma of the healthy skeletal muscle cells, and the occupied sarcoplasm.

PNA stained the cuticle, stychosome and hypodermis of *Trichinella*; the worm did not react with MAL (Fig. 1).

During the whole process of transformation and within the mature Nurse cell, the areas of the occupied sarcoplasm were

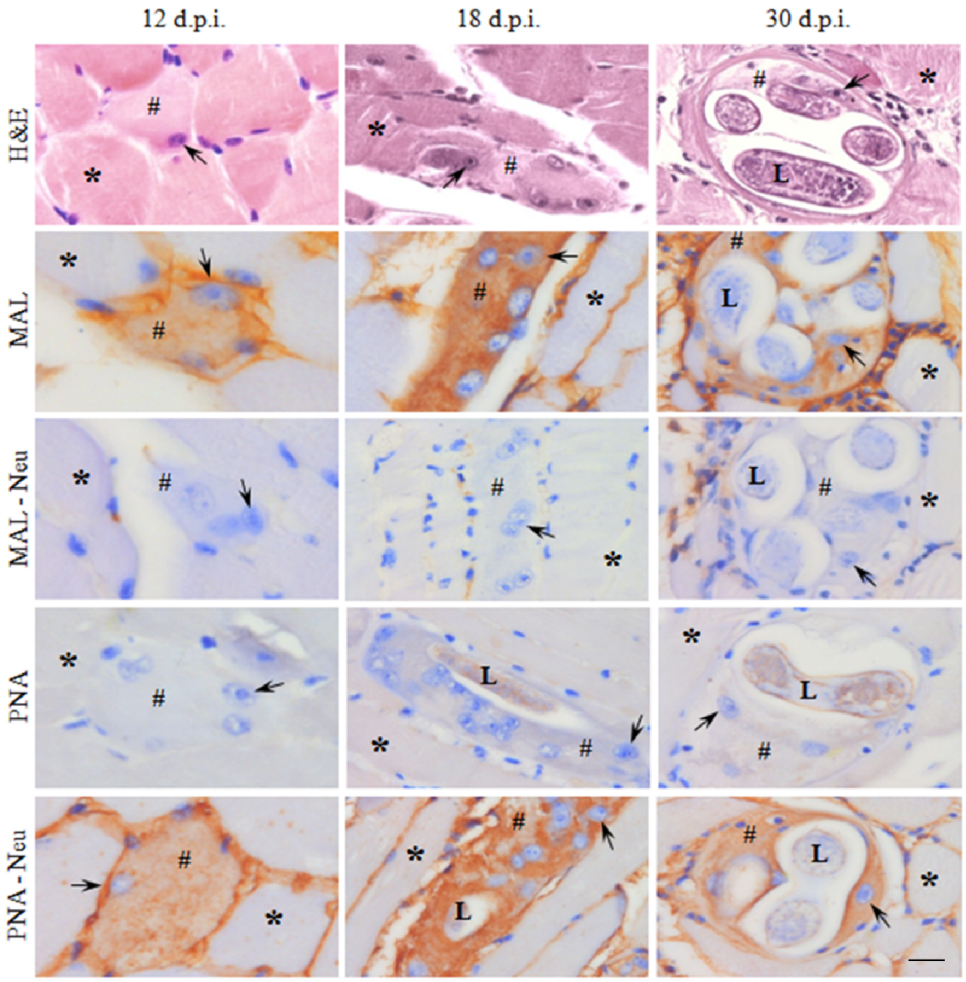

Fig. 1. Lectin histochemistry. Neutral buffered formalin fixed sections from mouse skeletal muscles with *Trichinella spiralis* at days 12, 18 and 30 post invasion (d.p.i.) stained with biotinylated lectins MAL and PNA. Neuraminidase from *Clostridium perfringens* was applied to verify the specificity of MAL by cleavage of the α-2,3-sialic acid residues. MAL and PNA stained the sarcolemma of healthy muscle cells and the invaded portions of sarcoplasm. PNA resulted in positive staining only after neuraminidase (Neu) pretreatement of the sections. Paralel sections were stained with H&E to facilitate the histological interpretation. The brown colour indicates positive histochemical reaction, hash-tag (#) indicates the occupied sarcoplasma, asterisk (*)– non-occupied skeletal muscle cell, arrow – enlarged nucleus, L – larva. H&E, streptavidin-biotin peroxidase, DAB. Scale bar 20 µm.

reactive towards MAL and towards PNA after neuraminidase pretreatment (Table 3, Fig. 1).

Expression of sialyltransferases

ST3Gal2 and ST3Gal4 reacted intensely against the sarcoplasm of healthy skeletal

Table 3. Evaluation of the intensity of intracellular staining within the skeletal muscle cells occupied by *Trichinella spiralis* after application of lectins with different carbohydrate specificity, with and without pretreatment with neuraminidase (Neu). The results were interpreted as negative (–), weakly (+), moderately (++) and strongly (+++) positive

Lectin	Days post infection		
	12	18	40
MAL	+++	+++	+++
MAL-Neu	+	–	–
PNA	–	–	–
PNA-Neu	+++	+++	+++

muscle cells. A transient increase of the expression of both sialyltransferases was observed within the early areas of invasion at d.p.i. 12, followed by a complete absence of staining further. The expressions of both enzymes were significantly diminished in the mature Nurse cell (30 d.p.i.). The infectious larva of *T. spiralis* was reactive towards the ST3Gal2 antibody (Fig. 2).

DISCUSSION

The sialylation in skeletal muscles has not been a subject of a profound research interest so far, even though the essential role of the sialic acids for the functional and structural maintenance of the skeletal muscle cells had been proven by many studies (Schwetz *et al.,* 2001; McDearmon *et al.,* 2003; Johnson *et al.,* 2004; Combs & Ervasti, 2005; Broccolini *et al.,* 2008; Hanish *et al.,* 2013). Histological expressions of sialic acids in human adult skeletal muscles were recently reported (Marini *et al.,* 2014). However, the α-dystroglycan, a crucial member of the dystrophin-associated glycoprotein complex, is the only muscular glycoprotein known to be sialylated (Baresi & Campbell, 2006). Except of one pilot research (Milcheva *et al.* 2018), detailed information about the expression of sialyltransferases in striated muscles is also unavailable.

It is already well known that the newborn larvae of *T. spiralis* induce a genetic reprogramming restricted only to the occupied portion of the host muscle cell, by suspension in apparent G2/M phase, change in expression of at least 55 genes and activation of collagen synthesis and angiogenesis. The enlarged nuclei are a permanent characteristic in all stages of development of the Nurse cell and remain transcriptionally active throughout the whole life of the Nurse cell, or until reinvasion occurs (Patra & Sarkar, 2014).

Previous studies of ours have demonstrated for the first time that the muscular phase of trichinellosis and the development of the Nurse cell were associated with increased intracellular accumulation of sialylated glycoproteins, localised within the invaded portions of muscle cells (Milcheva *et al.,* 2015; 2019). The current results suggested synthesis of sialyl-T-antigen (SiA-α-2,3-Gal-β-1,3-GalNAc-) and α-2,3-sialyllactosamine structure (SiA-α-2,3-Gal-β-1,4-GlcNAc). The sialyl-T-antigen is an O-linked glycan structure, and the α-2,3-sialyllactosamine is a part of hybrid or complex types of N-linked glycans (Brockhausen & Stanley, 2017; Stanley *et al.,* 2017). The presence of sialyl-T-antigen and 2,3-sialyllactosamine in the cytoplasm of the developing Nurse cell implies ST3Gal2 and ST3Gal4 enzyme activity (Kitigawa & Paulson, 1994; Kim *et al.,* 1996). Both enzymes belong to the β-galactoside-α-2,3-sialyltransferase family, that consists of two subfamilies, according to their specificity (Takashima, 2008). The mem-

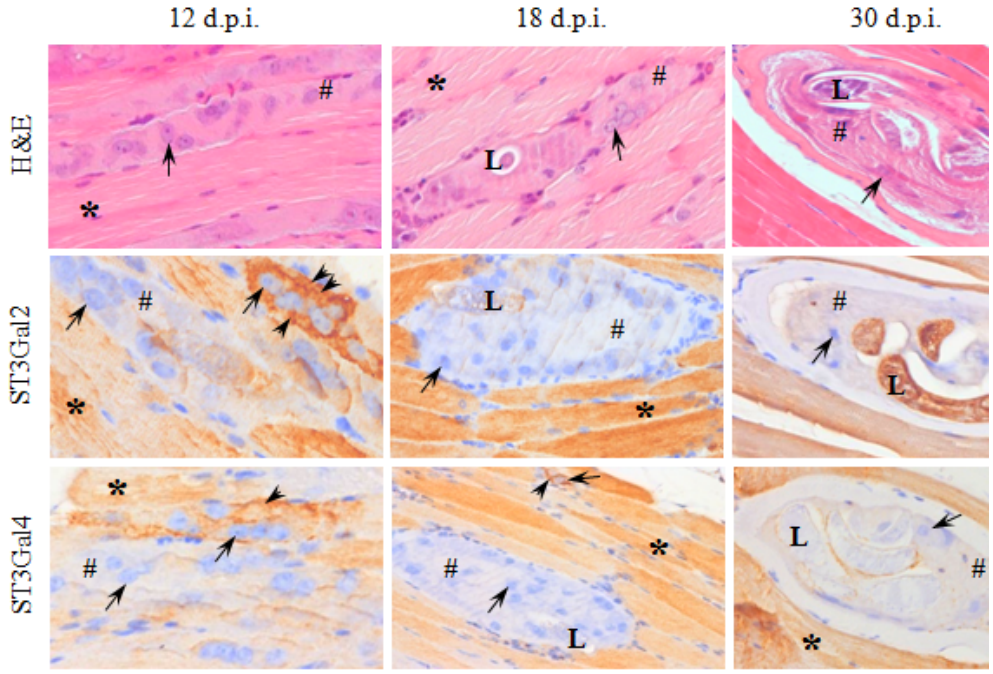

Fig. 2. Immunohistochemistry. Neutral buffered formalin fixed sections from mouse skeletal muscles with *Trichinella spiralis* at days 12, 18 and 30 post invasion (d.p.i.) were stained with rabbit polyclonal antibodies against ST3Gal2 and ST3Gal4 α-2,3-sialyltransferases. Paralel sections were stained with H&E to facilitate the histological interpretation. Both enzymes were strongly expressed in healthy muscle cells (star). After transient increase of expression of ST3Gal2 and ST3Gal4 at the beginning of invasion (arrows), the expression of both enzymes significantly diminished during the development of the Nurse cell. The brown colour indicates positive immunohistochemical reaction, hashtag (#) indicates the occupied sarcoplasm, asterisk (*) – non-occupied skeletal muscle cell, arrow – enlarged nucleus, arrowhead – transient increase of the expression within the occupied sarcoplasm, L – larva. H&E, streptavidin-biotin peroxidase, DAB. Scale bar 20 µm.

bers of the first subfamily, ST3Gal1 and ST3Gal2, are responsible for the synthesis of the sialyl-T-antigen (Takashima, 2008). A previous study of ours reported their mRNA expressions in healthy skeletal muscles (Milcheva *et al.,* 2018); however the expression of ST3Gal2 seemed to be down-regulated during the formation of the Nurse cell. Three members of the second subfamily, ST3Gal3, ST3Gal4 and ST3Gal6, utilise Gal-β-1,4-GlcNAc and Gal-β-1,3-GlcNAc structures mainly on glycoproteins, with different preferences (Takashima, 2008). Their mRNA expressions were also described in healthy skeletal muscle (Milcheva *et al.* 2018), but the current results showed that the ST3Gal4 enzyme was down-regulated in the invaded muscle cells, too.

In conclusion, it was found that after *T. spiralis* invasion, the occupied portion of skeletal muscle cell responded with synthesis of presumable sialyl-T-antigen and α-2,3-sialyllactosamine structure, that remained accumulated within the developing Nurse cell. The enzymes ST3Gal2 and

ST3Gal4, which could be responsible for the sialylation of each of these structures however, were not present in the invaded muscle portions, although their expressions in the healthy surrounding tissue remained persistent. Our future work will be dedicated in detailed description of the β-galactoside-α-2,3-sialyltransferases expressions in the Nurse cell of *T. spiralis* which would facilitate the understanding about the remarkable adaptive properties of the skeletal muscles and their unique relation with this nematode.

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