DOI 10.1007/s10517-021-05131-5

Reactive Changes in Elements of Stromal-Vascular Differons of Dysferlin-Deficient Skeletal Muscles after Procaine Injection

O. N. Chernova[1,2], M. O. Mavlikeev[1,3], A. P. Kiyasov[4], I. Ya. Bozo[3], and R. V. Deev[1,5]

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 170, No. 11, pp. 646-650, November, 2020
Original article submitted on May 7, 2020

The study assessed reactivity of stromal-vascular skeletal muscle differons to acute chemical injury. Dysferlin-deficient Bla/J mice and the wild-type C57BL/6 mice were intramuscularly injected with 100 μl of 0.5% procaine solution. The middle segment of gastrocnemius muscle was taken on postsurgery days 2, 4, 10, and 14 for routine histological examination. To evaluate proliferation and vascularization, the paraffin sections were stained immunohistochemically with antibodies to α-smooth muscle actin and Ki-67. The connective tissue was stained according to Mallory. The study revealed diminished proliferative activity of stromal-vascular differons and decreased vascular density in muscles of Bla/J mice. Thus, mutations in the DYSF gene coding dysferlin down-regulate the reparation processes in all differons of skeletal muscle.

Key Words: *dysferlinopathy; knockout mice; skeletal muscles; chemical injury*

Dysferlin is a transmembrane protein of ferlin family of proteins that is encoded by the *DYSF* gene and performs the following functions: repair of damaged membranes, fusion of vesicles, cellular adhesion, and maintenance of intercellular signaling [3-5,10]. It is also implicated in reorganization of microtubules during myogenesis [3,6,13]. Mutations in *DYSF* gene provoke one of the clinical forms of dysferlinopathies known as inherited muscular dystrophies.

This disease is characterized with the weakness of limb muscles followed by atrophy and disability. Pathomorphologically, dysferlinopathy is characterized by the muscle fiber (MF) necrosis, leukocyte infiltration, fibrosis and skeletal muscle lipidosis [7,8]. In view of the rapidly developing methods of gene therapy of hereditary pathologies, a number of knockout lines of animals have been developed for the study of pathogenesis and drugs testing.

Previously, we described the features of reparative regeneration of skeletal muscles in mice with dysferlin deficiency focusing on response of rhabdomyogenic differon [2]. There are following morphogenetic phases of reparative regeneration in the skeletal muscles: MF necrosis, activation of myosatellite cells, proliferation and differentiation of myoblasts accompanied with formation of myosimplast [1]. Regeneration of skeletal muscles is accompanied by active angiogenesis [14]. However, expression of dysferlin is normally detected not only in MF, but also in endotheliocytes [13], leukocytes [12], and fibroblasts [11]. Consequently, deficiency in DYSF expression may also affect the stromal-vascular response, remodeling, and functional adaptation of regenerated muscle. Unveiling these processes is important for the study of pathomorphogenesis and the development of etiopathogenetic therapy.

Our aim was to assess the effect of dysferlin deficiency on the elements of stromal-vascular differons (a lineage of cells forming vascular and connective-

[1]I. I. Mechnikov North-Western State Medical University, Ministry of Health if the Russian Federation; [2]St. Petersburg Medical-Social Institute, St. Petersburg, Russia; [3]Research Institute of General Pathology and Pathophysiology, Moscow, Russia; [4]Kazan (Volga region) Federal University, Kazan, Republic of Tatarstan, Russia; [5]Institute of Human Stem Cells, Moscow, Russia. ***Address for correspondence:*** olgachernova92@yandex.ru. O. N. Chernova

tissue components in the skeletal muscles) in response to procaine damage in mice.

MATERIALS AND METHODS

For pathomorphological evaluation of reparation processes in skeletal muscles in response to acute injury, a chemical damage model was chosen because of ease of dosing and precise administration of a myotoxic agent. A bolus (100 μl) of 0.5% procaine (Renewal, series No. 421115) was injected into the medial head of right gastrocnemius muscle of Bla/J mice (n=16, experimental group) and C57BL/6 mice (n=16, control group) via cutaneous incision [9]. In the injection site, the skin was shaved and treated with 70% ethyl alcohol. Skin was cut, and myotoxic agent was injected with insulin syringe under visual control. Finally, the wound was sutured. The animals were narcotized with Zoletil and medetomidine. At each time point (on postinjection days 2, 4, 10, and 14), the mice (n=4) were euthanized with overdose of anesthetics. Intact muscles of mice of the same age not injected with procaine (day 0) served as negative control. The experimental protocols were approved by Local Ethics Committee of Institute of Fundamental Medicine and Biology, Kazan Federal University (protocol No. 2, May 5, 2015).

The specimens of gastrocnemius muscle were fixed in 10% buffered formalin and embedded into paraffin using STP420E tissue processor (Thermo Scientific) according to the standard protocol. The semithin sections (4 μ) were cut on an HM355S microtome (Thermo Scientific). The sections were stained with hematoxylin and eosin or Mallory's trichrome. They were also stained immunohistochemically with antibodies (Ab) to α-smooth muscle actin (α-SMA) (1:50, clone 1A4, Dako) and Ki-67 (1:200, clone SP6; Abcam). The sections were used to study the histogenetic processes implicating the stromal-vascular differons of injured muscle tissue.

Proliferation was evaluated by immunohistochemical (IHC) staining with Ab to Ki-67. To this end, the ratio of Ki-67$^+$ nuclei in vascular endothelium to total number of nuclei in vascular endothelium and the ratio of Ki-67$^+$ nuclei in connective tissue (CT) to total number of nuclei in CT were calculated. Vascularization was evaluated by counting the vascular density (the ratio of number of vessels to MF) from sections stained immunohistochemically with Ab against α-SMA. The share of CT was assessed in sections stained with Mallory's trichrome.

The stained sections were photographed with an AxioImager Z2 light microscope (Carl Zeiss), thereupon they were scanned in an Aperio CS2 slide scanner (Leica Microsystems). The resulting images were analyzed using Aperio ImageScope and ImageJ. Statistical morphometry was performed on 10 microphotographs at ×400. The results were summarized as Me (Q1; Q3). The data were analyzed with Statistica 13.3 software (StatSoft, Inc.) employing Kruskal—Wallis test at p<0.05.

RESULTS

Previously, we revealed a diminished proliferative activity of myogenic cells in the absence of dysferlin expression [9]. The levels and dynamics of the share of proliferating stromal cells were almost identical to those in muscular component described earlier. In mice of both lines, the proliferative activity of stromal cells gradually decreased to experimental day 14. This activity was smaller in Bla/J mice at all time points except for day 10 (Fig. 1) being 4.34 (2.70; 6.52)% *vs* 0 (0; 2.69)% in C57BL/6 mice (p<0.05). The decline of proliferative activity accelerated on days 2-4 in Bla/J mice and on days 4-10 in C57BL/6 line. This observation indicates a less intense and later completion of proliferation in Bla/J mice in comparison with C57BL/6 line. By day 14, the indicators of proliferative activity virtually dropped to zero in both groups and approached to those of negative control.

Unlike stromal and muscular cells, the endotheliocytes did not demonstrate a gradual decrease of proliferation activity neither in control nor in experimental group. However, the proliferation activity was lower in Bla/J mice from days 4 to 14, which confirms the hypothesis of diminished proliferative activity in the absence of dysferlin, including this activity in vascular component of skeletal muscles (Fig. 2). In control group, proliferation activity increased between days 2 and 4 from 10.72 (0; 21.58) to 28.23 (20.92; 35.84)% (p<0.05), whereas in experimental group, it increased only from 13.33 (11.76; 26.79) to 15.38 (11.11; 22.22)% (p<0.05). Therefore, the dysferlin-deficient mice possess an insufficient reserve for proliferation of endotheliocytes. Probably, this deficiency results from the absence of dysferlin in endotheliocytes where its involvement in angiogenesis was established [4].

Remodeling and functional adaptation are the final steps in restoring morphofunctional ability of the damaged tissue as evidenced by fibrosis and vascularization. Here, the vascular density value increased in both groups starting from day 2, and it reached the maxima on day 14 counting 22.22 (18.42; 26.09)% in the control group and 19.61 (15.64; 24.00)% in experimental one (p=0.09, Fig. 3). A small vascularization ratio in the experimental group can be associated with a low expression of dysferlin in endotheliocytes. Probably, a reduced vascularization in the absence of

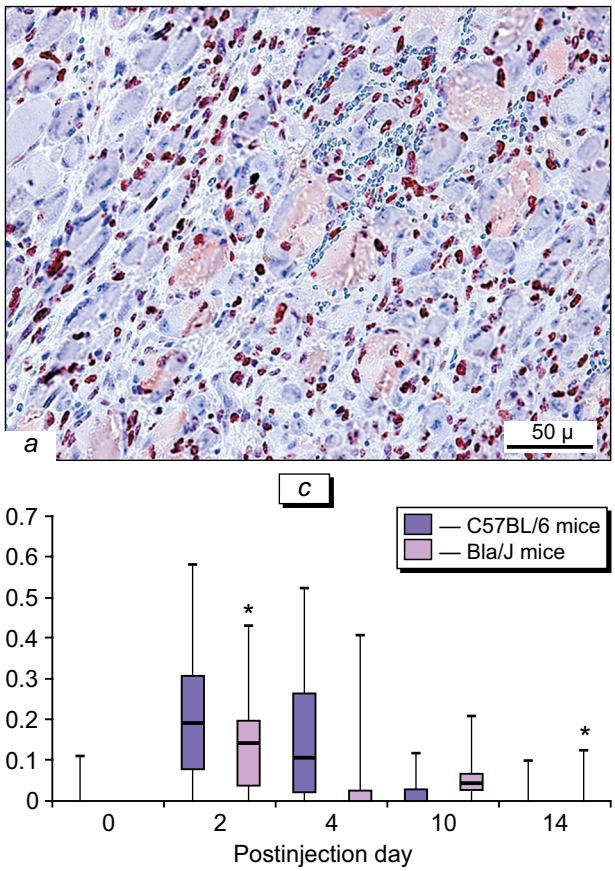

Fig. 1. Sections of gastrocnemius muscles of C57BL/6 (*a*) and Bla/J (*b*) mice on day 4 after procaine injection, and Ki-67$^+$ nuclei ratio in stroma (*c*). Staining with Ab to Ki-67, ×400 (*a*, *b*). *p<0.05 in comparison with C57BL/6 mice.

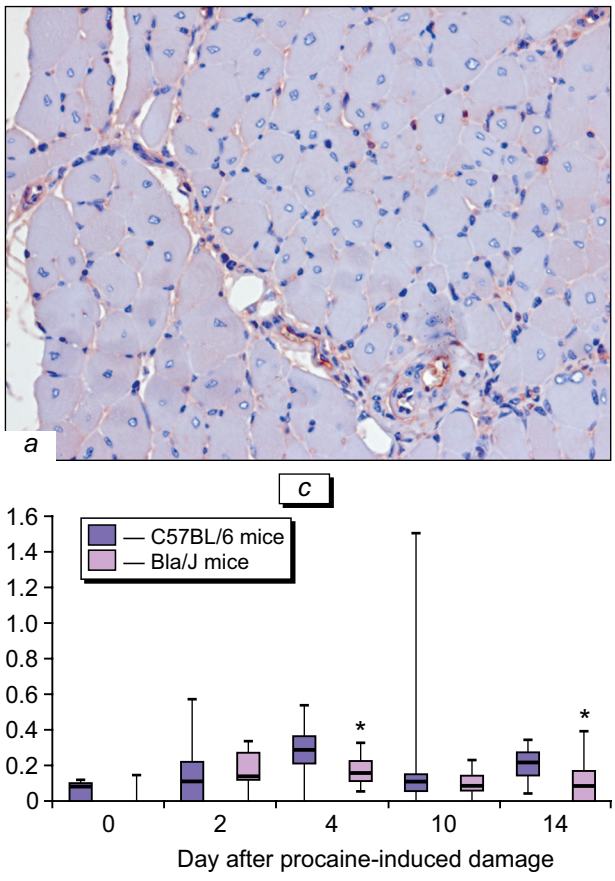

Fig. 2. Sections of gastrocnemius muscles of C57BL/6 (*a*) and Bla/J (*b*) mice on day 14 after procaine injection, and Ki-67$^+$ nuclei ratio in endotheliocytes (*c*). Staining with Ab to Ki-67, ×400 (*a*, *b*). *p<0.05 in comparison with C57BL/6 mice.

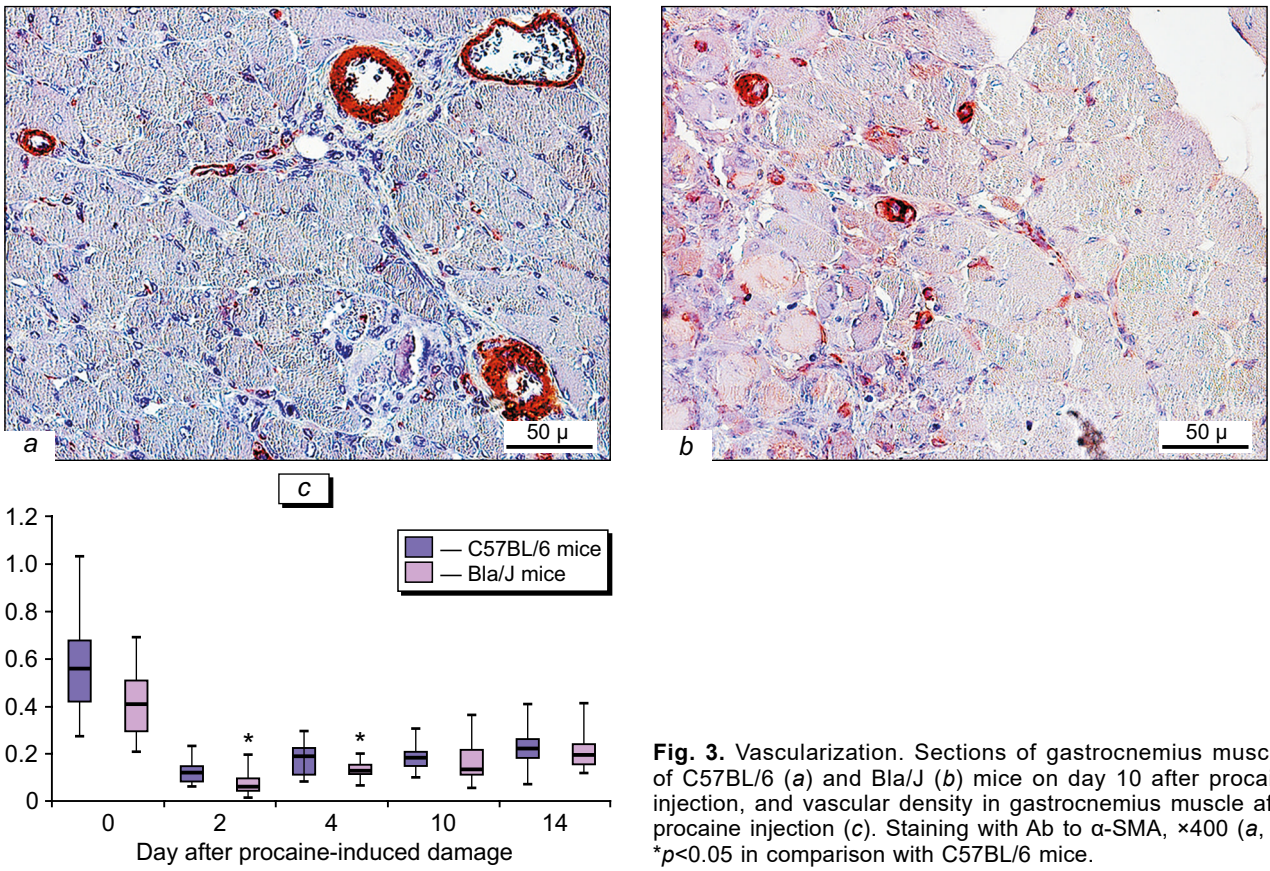

Fig. 3. Vascularization. Sections of gastrocnemius muscles of C57BL/6 (*a*) and Bla/J (*b*) mice on day 10 after procaine injection, and vascular density in gastrocnemius muscle after procaine injection (*c*). Staining with Ab to α-SMA, ×400 (*a*, *b*). *$p<0.05$ in comparison with C57BL/6 mice.

dysferlin leads to hypoxia in the tissue, which down-regulates the reparative activity in skeletal muscles.

The differences in proportion of CT were statistically significant at all time points, but the maximum value was attained in Bla/J mice on day 10 (0.06 (0.01; 0.12)%). Thus, the absence of dysferlin does not affect the fibrotic processes under the condition of acute responsiveness of skeletal muscle.

The study showed that regenerative histogenesis in dysferlin-deficient muscles is characterized with alterations not only in MF, but also in vascular-stromal differons. The study revealed a smaller proliferative activity of stromal cells in Bla/J mice in comparison with that in C57BL/6 mice. Similarly, on day 14, the proliferative activity of endotheliocytes in dysferlin-deficient mice was smaller than that in wild-type mice. At all time points, vascularization was also lower in Bla/J mice compared to the control ones. Thus, during acute damage to skeletal muscles, dysferlin is implicated in the recovery processes, which are developing not only in MF, but also in other cellular elements of the muscle.

The authors are grateful to Prof. A. N. Tomilin for the generous gift of mice.

This work was supported by the Russian Science Foundation (grant No. 18-75-10085) and carried out within the Federal Support Program aimed to enhance competition of Kazan Federal University. It was also supported with subsidies granted to Kazan Federal University for the implementation of State Task in Research Field.

REFERENCES

1. Histology Guideline. Danilov RK, ed. St. Petersburg, 2011. Vol. 1. P. 425-490. Russian.
2. Chernova ON, Mavlikeev MO, Zeynalova AK, Kiyasov AP, Deev RV. Reparative rhabdomyogenesis in mice with DYSF mutation. Geny Kletki. 2019;14(2):32-39. Russian.
3. Azakir BA, Di Fulvio S, Therrien C, Sinnreich M. Dysferlin interacts with tubulin and microtubules in mouse skeletal muscle. PLoS One. 2010;5(4):e10122. doi: 10.1371/journal.pone.0010122
4. Bansal D, Campbell KP. Dysferlin and the plasma membrane repair in muscular dystrophy. Trends Cell Biol. 2004;14(4):206-213.
5. de Morrée A, Flix B, Bagaric I, Wang J, van den Boogaard M, Grand Moursel L, Frants RR, Illa I, Gallardo E, Toes R, van der Maarel SM. Dysferlin regulates cell adhesion in human monocytes. J. Biol. Chem. 2013;288(20):14 147-14 157.
6. Di Fulvio S, Azakir BA, Therrien C, Sinnreich M. Dysferlin interacts with histone deacetylase 6 and increases alpha-tubulin acetylation. PLoS One. 2011;6(12):e28563. doi: 10.1371/journal.pone.0028563

7. Fanin M, Angelini C. Muscle pathology in dysferlin deficiency. Neuropathol. Appl. Neurobiol. 2002;28(6):461-470.
8. Gallardo E, Rojas-García R, de Luna N, Pou A, Brown RH Jr, Illa I. Inflammation in dysferlin myopathy: immunohistochemical characterization of 13 patients. Neurology. 2001;57(11):2136-2138.
9. Hardy D, Besnard A, Latil M, Jouvion G, Briand D, Thépenier C, Pascal Q, Guguin A, Gayraud-Morel B, Cavaillon JM, Tajbakhsh S, Rocheteau P, Chrétien F. Comparative study of injury models for studying muscle regeneration in mice. PLoS One. 2016;11(1):e0147198. doi: 10.1371/journal.pone.0147198
10. Kerr JP, Ziman AP, Mueller AL, Muriel JM, Kleinhans-Welte E, Gumerson JD, Vogel SS, Ward CW, Roche JA, Bloch RJ. Dysferlin stabilizes stress-induced Ca2-signaling in the transverse tubule membrane. Proc. Natl. Acad. Sci. 2013;110(51):20831-20836.
11. Matsuda C, Kiyosue K, Nishino I, Goto Y, Hayashi YK. Dysferlinopathy fibroblasts are defective in plasma membrane repair. PLoS Curr. 2015;7. pii: ecurrents.md.5865add2d766f39a0e0411d38a7ba09c. doi: 10.1371/currents.md.5865add2d766f39a0e0411d38a7ba09c
12. Nagaraju K, Rawat R, Veszelovszky E, Thapliyal R, Kesari A, Sparks S, Raben N, Plotz P, Hoffman EP. Dysferlin deficiency enhances monocyte phagocytosis: a model for the inflammatory onset of limb-girdle muscular dystrophy 2B. Am. J. Pathol. 2008;172(3):774-785.
13. Sharma A, Yu C, Leung C, Trane A, Lau M, Utokaparch S, Shaheen F, Sheibani N, Bernatchez P. A new role for muscle repair protein dysferlin in Endothelial cell adhesion and angiogenesis-R2. Arterioscler. Thromb. Vasc. Biol. 2010;30(11):2196-2204.
14. Warren GL, Summan M, Gao X, Chapman R, Hulderman T, Simeonova PP. Mechanisms of skeletal muscle injury and repair revealed by gene expression studies in mouse models. J. Physiol. 2007;582(Pt 2):825-841.