J Toxicol Pathol 2020; 33: 115–119

Original Article

Pathological study of tubular aggregates occurring spontaneously in the skeletal muscles of non-obese diabetic/Cg-*Prkdc^scid^Il2rgt^m1sug^*/ ShiJic (NOG) mice

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Abstract: To examine the biological and morphological features of tubular aggregates (TAs) in the skeletal muscles of non-obese diabetic/Cg-*Prkdc^{scid}Il2rgt^{m1Sug}*/ShiJic (NOG) mice, 73 male and 72 female specific-pathogen-free NOG mice were examined at 7, 18, 22, 26, and 52 weeks of age. TAs were observed as intracytoplasmic eosinophilic materials of the femoral muscles in males at 18, 22, 26, and 52 weeks of age and in females at 52 weeks of age; gender-related differences were noted in the onset time and lesion degree. Intracytoplasmic materials were positive for Gomori's trichrome stain. Electron microscopy revealed that TAs were composed of an accumulation of dilated sarcoplasmic reticulum. In addition, TAs were observed in the femoral and gastrocnemius muscles, but not in the soleus and diaphragm muscles, suggesting that TAs are present in fast muscle fibers. The morphology of TAs and the type of myofibers involved, as well as the gender difference in NOG mice were essentially the same as those of TAs observed in C57BL/6J and MRL+/+ mice. (DOI: 10.1293/tox.2019-0079; J Toxicol Pathol 2020; 33: 115–119)

Key words: tubular aggregate, non-obese diabetic/Cg-*Prkdc^{scid}Il2rgt^{m1Sug}*/ShiJic (NOG) mouse, skeletal muscle, sarcoplasmic reticulum

Introduction

Non-obese diabetic (NOD)/Cg-*Prkdc^{scid}Il2rgt^{m1Sug}*/ ShiJic (NOG) mice are the latest and most versatile severely immuno-deficient animals for human cell/tissue transplantation studies. NOG mice were established at the Central Institute for Experimental Animals (Kanagawa, Japan) by knockout introduction of the IL-2 receptor γ-chain (IL2Rγc) into the NOD-scid strain[1]. NOG mice are characterized by deficient T-, B-lymphocytes and natural killer cells, and reduced function of macrophages and dendritic cells[1]. Since human cells and tissues can integrate more efficiently into NOG mice in comparison with other immune-deficient mice such as nude mice, they are considered a useful evaluation system for *in vivo* tumorigenicity studies to examine the tumorigenic potential of human pluripotent stem cells[1].

Tubular aggregates (TAs) have been reported to be accompanied by muscle weakness and occur in the skeletal muscle of several myopathies, such as periodic paralysis, myasthenic syndrome and myotonic disorders in human[2]. TAs have been shown to be an accumulation of dilated sarcoplasmic reticulum[3]. Such changes also occur in male MRL+/+ mice and both genders of ICR mice[4, 5]. In our previous study, we reported histopathological background data on untreated NOG mice that were housed in cages installed in a positive pressure rack up to 52 weeks of age[6]. Among the lesions observed in these mice, we identified TAs in skeletal muscles in males at 26 and 52 weeks of age and females at 52 weeks of age; gender differences in the onset time and lesion degree were also observed. In the present study, we have conducted further examination on the onset and gender differences of TAs, and more detailed histopathological examination as to which type of muscle fibers were susceptible.

Materials and Methods

Animals

Seventy-three male and 72 female specific-pathogen-free NOG mice were obtained from the Central Institute for Experimental Animals (Kanagawa, Japan).

Animal husbandry

All animals were housed in cages in a positive pressure rack in a clean animal room controlled to maintain the

Received: 30 September 2019, Accepted: 26 December 2019
Published online in J-STAGE: 25 January 2020
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temperature at 23 ± 3°C, relative humidity at 50 ± 20%, air ventilation at 10 to 20 times per hour, and illumination for 12 h/day. In this rack, each male and five females/cage were reared in a polycarbonate flat-bottomed cage for single-housing (W 160 × D 370 × H 130 mm, Tecniplast Japan Co., Ltd., Tokyo, Japan) and polycarbonate flat-bottomed cages for group-housing (W 230 × D 335 × H 140 mm, CLEA Japan Inc., Tokyo, Japan), respectively. Mice were allowed free access to a pelleted diet, CE-2 (irradiation sterilized, CLEA Japan Inc.), and tap water. The equipment was disinfected with hypochlorous acid or by autoclaving. Moreover, traffic lines for humans and equipment to access to each animal room were strictly controlled.

Animal welfare

This experiment was conducted after obtaining approval of the Animal Experiment Committee of BoZo Research Center Inc. (Tsukuba, Japan) in 2015 (APS14017). It was conducted in accordance with the guidelines for the control and welfare of experimental animals specified by the test facility (Rules of the Animal Experiment Committee, BoZo Research Center Inc.).

Histology

The animals were kept untreated until euthanasia under isoflurane anesthesia at 7, 18, 22, 26, or 52 weeks of age. The femoral muscles of both genders at 7, 18, 22 (only males), 26, and 52 weeks of ages, and the gastrocnemius, soleus and diaphragm muscles of both genders at 52 weeks of ages were collected and examined histopathologically.

Skeletal muscles were fixed in 10% phosphate-buffered formalin. These tissues were trimmed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). Moreover, femoral muscles of males at 22 weeks of age were immediately frozen in cooled acetone. Ten micrometer-thick cryostat sections were stained with modified Gomori's trichrome stain and HE.

Electron microscopy

Small pieces of the formalin-fixed femoral muscle from three males at 7, 26, and 52 weeks of age and one female at 52 weeks of age were additionally fixed in a solution of phosphate buffered 0.5% glutaraldehyde plus 1.5% paraformaldehyde, then post-fixed in 1% osmium tetroxide solution, and embedded in epoxy resin. Ultra-thin sections were prepared, stained with uranyl acetate and lead citrate, and observed under electron microscopy (JEM-1400, JEOL Ltd., Tokyo, Japan).

Results

Histology

Femoral muscles (Table 1): Accumulation of intracytoplasmic eosinophilic materials was observed in the femoral muscle of all males at 18, 22, 26, and 52 weeks of age, and increased in size and number at 52 weeks of age (Fig. 1 and 2). In females, these aggregates were observed in the femoral muscle of all animals at 52 weeks of age, but not at 18 and 26 weeks of age. The severity of lesions in females at 52 weeks of age was less than that in males (Fig. 3). Intracytoplasmic eosinophilic materials were positively stained (red) with Gomori's trichrome stain in males at 22 weeks of

Table 1. Distribution of Eosinophilic Materials in Various Muscles at Different Ages of Non-obese Diabetic/Cg-*Prkdc*^{scid}*Il2rg*^{tm1sug}/ShiJic (NOG) mice

Observation		Male					Female			
	Sex :	Male					Female			
	Weeks of age :	7	18	22	26	52	7	18	26	52
	Number of animals :	20	10	4	20	19	20	5	28	19
HE stain										
Femoral muscle	Number examined	20	10	4	20	19	20	5	28	19
	Eosinophilic materials	0	10	4	20	19	0	0	0	19
	minimal	0	10	4	20	5	0	0	0	19
	mild	0	0	0	0	14	0	0	0	0
Gomori's trichrome stain										
Femoral muscle	Number examined	NE	NE	4	NE	NE	NE	NE	NE	NE
	Red materials	NE	NE	4	NE	NE	NE	NE	NE	NE
	present	NE	NE	4	NE	NE	NE	NE	NE	NE
HE stain										
Gastrocnemius muscle	Number examined	NE	NE	NE	NE	16	NE	NE	NE	16
	Eosinophilic materials	NE	NE	NE	NE	16	NE	NE	NE	0
	minimal	NE	NE	NE	NE	16	NE	NE	NE	0
Soleus muscle	Number examined	NE	NE	NE	NE	18	NE	NE	NE	18
	Eosinophilic materials	NE	NE	NE	NE	0	NE	NE	NE	0
Diaphragm	Number examined	NE	NE	NE	NE	17	NE	NE	NE	18
	Eosinophilic materials	NE	NE	NE	NE	0	NE	NE	NE	0

NE: Not examined.

age (Fig. 4). However, there were no reactive changes such as degeneration/necrosis of muscles and inflammation related to TAs.

Gastrocnemius muscles (Table 1): Intracytoplasmic eosinophilic materials were observed in the gastrocnemius muscle of all males at 52 weeks of age.

Soleus muscles (Table 1): Intracytoplasmic eosinophilic materials were not observed in the soleus muscle of both genders at 52 weeks of age.

Diaphragms (Table 1): Intracytoplasmic eosinophilic materials were not observed in the diaphragm of both genders at 52 weeks of age.

Electron microscopic examination

Electron microscopic examination revealed that these intracytoplasmic materials consisted of an accumulation of dilated sarcoplasmic reticulum. This change was observed in a male at 26 and 52 weeks of age and in a female at 52 weeks of age (Fig. 5 and 6), and the severity of this change increased at 52 weeks of age. In addition, high electron

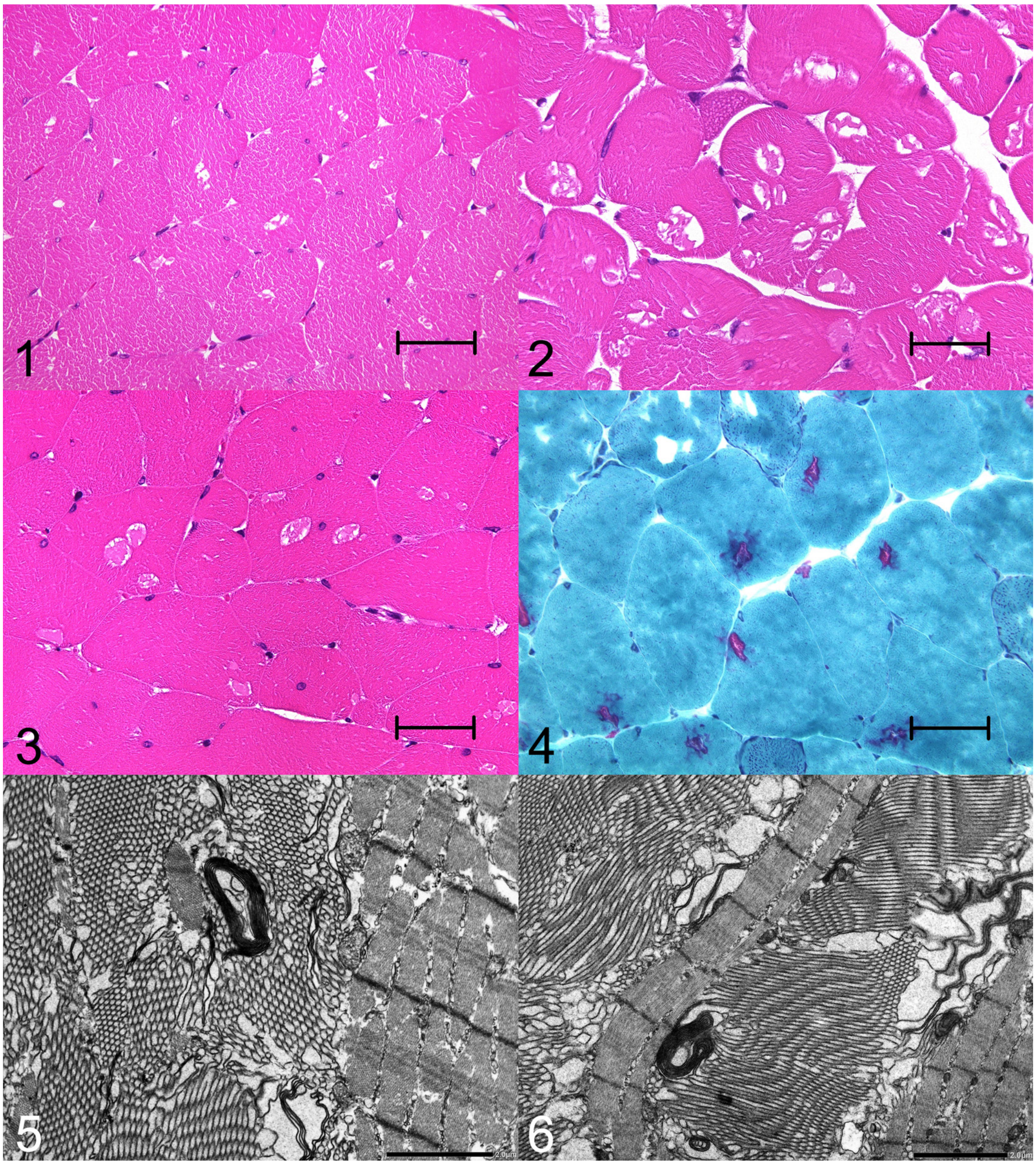

Fig. 1. Minimal intracytoplasmic eosinophilic materials in the femoral muscle of a male at 18 weeks of age. HE stain, Bar=50 µm.
Fig. 2. Mild intracytoplasmic eosinophilic materials in the femoral muscle of a male at 52 weeks of age. HE stain, Bar=50 µm.
Fig. 3. Minimal intracytoplasmic eosinophilic materials in the femoral muscle of a female at 52 weeks of age. HE stain, Bar=50 µm.
Fig. 4. Red materials in the femoral muscle of a male at 22 weeks of age. Gomori's trichrome stain. Bar=50 µm.
Fig. 5. Accumulation of dilated sarcoplasmic reticulum in the femoral muscle of a male at 52 weeks of age. Electron micrograph. Bar=2 µm.
Fig. 6. Accumulation of dilated sarcoplasmic reticulum in the femoral muscle of a female at 52 weeks of age. Electron micrograph. Bar=2 µm.

dense materials were observed in muscle fibers of a male at 52 weeks of age. The arrangement of muscle fibers containing accumulated sarcoplasmic reticulum showed an irregular pattern.

Discussion

Intracytoplasmic eosinophilic materials observed in this study were positively stained with Gomori's trichrome that is used for the demonstration of collagen fibers. Electron microscopic examination revealed that these intracytoplasmic materials were composed of an accumulation of dilated sarcoplasmic reticulum, known as TAs in muscle cells in humans and mice[2].

In NOG mice, TAs were observed in all females at 52 weeks of age, but the onset time of TAs in females was delayed as compared with that in males and the severity of the lesions in females was lower than that in males. In inbred mice such as C57BL/6J and MRL+/+mice, TAs occur only in males[2]. However, the spontaneous occurrence of TAs was reported to occur in both genders of ICR mice more than 7 months of age, but the occurrence of TAs in female ICR mice was 2 out of 20 and markedly less than that in males (26 out of 26)[5]. Therefore, there are gender differences in the occurrence of TAs in mice, and it has been pointed out that the male sex hormone is probably involved in the occurrence of TAs in mice[2, 5]. Gender differences in the NOG mice suggest that the male sex hormone is probably involved in the onset of TAs in NOG mice, as in other strains of mice. In females, since TAs were observed in ICR mice (outbred strain in a closed colony) and NOG mice but not in inbred mice, some kinds of unknown differences in genetic factors were also thought to be related to the difference in the incidence of TAs between NOG mice and other inbred mice.

TAs were observed in the femoral and gastrocnemius muscles in NOG mice, but not in the soleus and diaphragm muscles. These findings were similar to those observed in other inbred strains of mice[2]. In inbred strains of mice, TAs are found in the femoral and gastrocnemius muscles, which are composed of abundant type IIB muscle fibers, but not in the soleus muscles composed of type I and type IIA muscle fibers[2, 7]. The above findings suggest that TAs in NOG mice occur in fast muscle fibers, as in other inbred strains of mice.

The morphology of TAs and the type of myofibers involved, as well as the gender difference in NOG mice, were similar to those in other strains of mice. Therefore, it was suggested that TAs observed in NOG mice are essentially the same as those in other strains of mice. However, Agbulut *et al.*[2] pointed out that since TAs are never observed in female inbred mice and are only found in type IIB glycolytic muscle fibers of male inbred mice, TAs are a non-specific phenomenon induced by inbreeding.

High electron dense materials were observed in muscle fibers of a male at 52 weeks of age. The high electron dense materials in this study morphologically resemble nemaline rods in mice with congenital myopathy[8]. However, there have been no reports demonstrating that these electron dense materials were detected in mice with TAs. In this study, the association between TAs and high electron dense materials observed in NOG mice could not be clarified.

It has been shown that TAs in aging mice include the proteins sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA 1), sarcalumenin (longitudinal sarcoplasmic reticulum), calsequestrin (terminal cisternae) and ryanodine receptor (RyR: junctional sarcoplasmic reticulum), as well as 95 and 51 kDa isoforms of triadin[9]. In addition, Boncompagni *et al.*[10] reported that since calsequestrin accumulates in the sarcoplasmic reticulum and causes swelling of the sarcoplasmic reticulum in wild-type mice, TAs are probably deposit sites of the accumulated protein. In humans, there is a report suggesting that a mutation of stromal interaction molecule 1 (STIM1) results in dysregulation of Ca^{2+} homeostasis followed by the appearance of TAs[11]. It was suggested that TAs of NOG mice were essentially the same as those in other strains of mice. Therefore, TAs in NOG mice might be caused by the same mechanism as in aging mice. However, other reports indicated that homeostasis of Ca^{2+} was not involved in the formation of mouse TAs[9, 10]. Since TAs were observed in all types of muscle fibers in humans of both genders, TAs in mice may be induced by another mechanism that is different from that in humans[3]. Thus, the results of our study indicated that the appearance of TAs in NOG mice was different from that in humans in terms of the type of myofibers and gender involved.

In conclusion, TAs in NOG mice were microscopically observed as intracytoplasmic eosinophilic materials. These intracytoplasmic materials were positively stained with Gomori's trichrome. In addition, an electron microscopic examination revealed that they were composed of an accumulation of dilated sarcoplasmic reticulum. TAs in NOG mice were observed in both genders and the severity increased with age, but the severity of lesions in females was less than that in males. The results of the present study and previous studies suggest the possibility that TAs in NOG mice are a non-specific phenomenon induced by inbreeding and the male sex hormone is involved in the occurrence of TAs. Further studies are necessary to elucidate the biological features of TAs in the skeletal muscles of mice.

Disclosure of Potential Conflicts of Interest: The authors declare that there is no conflict of interest.

Acknowledgments: The authors gratefully acknowledge Dr. Kunitoshi Mitsumori, Professor Emeritus of the Tokyo University of Agriculture and Technology, for critical review of the manuscript and the Central Institute for Experimental Animals for providing the NOG mice.

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