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Mdx respiratory impairment following fibrosis of the diaphragm

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Abstract

Duchenne muscular dystrophy (DMD) is a progressive muscle-wasting disease that causes respiratory or cardiac failure and results in death at about 20 years of age. An animal model of DMD, the *mdx* mouse, is commonly used to estimate dystrophic pathology. The pathological features of limb muscles are relatively mild, however the diaphragm is severely affected and exhibits a degenerative pattern similar to that observed in human DMD. Although, the muscle strength assay of the dystrophic diaphragm has been used to estimate *mdx* respiratory impairment, systemic functional assessments compared with histopathological analysis have not been demonstrated. Here, we report a sensitive procedure using whole-body plethysmography to monitor respiratory parameters detected during early respiratory insufficiency in the *mdx* mouse. The dystrophic changes in the diaphragm lead to respiratory dysfunctions. These methods may be useful to assess the therapeutic approaches for the *mdx* mouse.

Keywords: Duchenne muscular dystrophy; *Mdx* mouse; Respiratory function; Whole-body plethysmography; Gene therapy

1. Introduction

Duchenne muscular dystrophy (DMD) and the milder allelic Becker muscular dystrophy (BMD) are X-linked genetic disorders. DMD is caused by the absence of dystrophin [1,2], a 427-kDa protein encoded on the short arm of the X chromosome by the largest gene currently known [3]. Affected boys are usually diagnosed between 3 and 5 years of age [4]. Early symptoms of delayed walking and gait disturbance rapidly progress to general muscle weakness, especially in proximal musculature. By age 12, 95% of patients are confined to a wheelchair and most have developed severe scoliosis. Even though improved clinical management has extended the life expectancy of DMD patients in recent years, most of the patients still die around age 20 from respiratory and/or cardiac failure [5]. Current treatment options for DMD patients focus primarily on the relief of symptoms, since no cure has been found for patients with DMD. The lack of dystrophin expression compromises the structural integrity of the muscle fiber membrane and renders muscles more susceptible to contraction-mediated injury and degeneration [1,6,7]. As a consequence, dystrophic muscle fibers continually undergo degeneration only to be replaced by regenerating fibers with the same genetic deficiency and injury susceptibility. For assessment of the disease progression stage, clinicians examine the skeletal muscle power of the extremities and the trunk, as well as the maximal respiratory pressure and endurance, which assesses the diaphragm and other respiratory muscles.

The *mdx* mouse, an animal model for DMD, carries a mutation in the dystrophin gene and lacks the native protein similar to the human condition, but exhibits a more benign pathological phenotype than human DMD patient. However, similar to the DMD patient, the reduced life span of *mdx* mice is considered to result primarily from

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respiratory and/or cardiac failure [8]. The diaphragm muscle of *mdx* mouse exhibits progressive structural and functional deterioration consistent with DMD, whereas the limb muscle show relatively mild pathology for much of the life span [9,10]. Therefore, the diaphragm of the *mdx* mouse may provide a more sensitive screen for therapeutic approaches.

Recent advances have identified many possible therapeutic approaches including pharmacological treatments [11] such as the use of myostatin antibodies [12,13], gene therapies and cell therapy with different types of newly identified stem cells [14]. In particular, viral vector mediated gene therapies with the delivery of therapeutic *micro-dystrophin* [15–18], *mini-dystrophin* genes [19], or full-length dystrophin cDNA [20,21] have been shown to reduce dystrophic changes in neonatal and young adult *mdx* mice. In some of these studies, the contractile property of the *mdx* diaphragm was measured to assess the correction of muscle function, as well as pathological improvements. As an alternative, whole-body plethysmography has been developed to assess respiratory function in the small animal model, especially mouse models of respiratory dysfunctions [22,23]. The latest version of this system can easily monitor some parameters of respiratory function without any restriction or anesthesia [24].

The aim of this study was to examine the use of whole-body plethysmography in assessing respiratory dysfunction in *mdx* mice. We show that this sensitive and systemic assay method detects a significant decrease of tidal volume in adult *mdx* mice compared with age matched wild type control mice, and that this physiological respiratory dysfunction correlates with diaphragm degeneration at an early stage of dystrophic and fibrotic pathology. This method may be useful to assess therapeutic improvement of candidate gene and cell therapies.

2. Materials and methods

2.1. Animal models

Two, 4, and 7 month-old-C57BL/10 *mdx* and C57BL/10 strains of mice (Central Institute for Experimental Animals, Kawasaki, Japan) were used in this study. All animal experiments in this study were approved by the Kumamoto University Committee on Animal Research. The mice were housed in the Center for Animal Resources and Development (CARD) of Kumamoto University in rooms that rooms were maintained in temperature (22 ± 2 °C) and relative humidity ($50 \pm 10\%$). The animals were housed under a 12/12 h light/dark cycle and had free access to drinking water and standard chow.

2.2. Histopathological analysis

At 2, 4, and 7 months of age, after the respiratory function test, the diaphragm of each mouse was carefully dissected and cryopreserved using Optimal Cutting Temperature compound (OCT; Sakura Finetechnical Co., Tokyo, Japan) and liquid nitrogen cooled isopentane (NACALAI TESQUE, INC., Kyoto, Japan). Muscle sections of 10 μm thickness were prepared using a cryostat. Each serial section was stained using the hematoxylin and eosin (H&E) and Masson trichrome methods. The following parameters were measured and evaluated, as previously described [18,25]: (1) percentage of centrally nucleated fibers and (2) the level of fibrosis were evaluated for dystrophic change with Masson trichrome staining. The area of fibrosis was calculated from the entire muscle cross-sectional area (%). Analysis was performed by WinROOF software (version 5.6, MITANI CORRPORATION, Fukui, Japan).

2.3. Immunohistochemistry

Immunostaining was performed as previously described [26]. The primary antibody for dystrophin was rabbit polyclonal anti-dystrophin antibody, dystrophinH-300 (Santa Cruz Biotechnology, Inc., 1:100) and the secondary antibody was Alexa546-labeled goat anti-rabbit IgG (H + L) (Molecular Probes, Eugene, OR, USA, 1:200). Stained sections were observed using a confocal laser scanning microscope (LSM410, Carl Zeiss Microscopy, Sena, Germany) and an optical microscope (DP70-WPCXP, Olympus, Tokyo, Japan).

2.4. Assessment of respiratory function

Mouse respiratory function was evaluated by whole-body plethysmography, as described previously [22,24]. This latest version of the non-invasive monitoring system provides robust pulmonary analysis that minimizes artifact from animal movement. Briefly, each unrestrained conscious mouse, 2, 4, and 7-months-old *mdx* and C57BL/10, was placed in a "free moving" chamber (450 ml, PLY3211, Buxco Electronics, Wilmington, NC, USA) (Fig. 3A), then monitored and analyzed by BioSystem XA software (Buxco Electronics, Wilmington, NC, USA) at stable temperature (22 ± 2 °C) and humidity ($50 \pm 10\%$). Each parameter, respiratory rate (RR), tidal volume (TV), peak inspiratory flow (PIF), minute volume (MV), and peak expiratory flow (PEF), Expiratory time (Te), relaxation time (RT), were recorded and analyzed in real time (Fig. 3B), then average values were calculated one per minute for each serial 10 min. Mean airway broncho-constriction was estimated by the Enhanced Respiratory Pause (Penh) index. Penh is calculated by the formula $\text{Penh} = (\text{Te}/\text{RT} - 1) \times \text{PEF}/\text{PIF}$.

2.5. Statistical studies

Data were expressed as means ± standard error of the mean. Statistical analysis was performed using a commercially available software package (StatView, Version 5, SAS Institute Inc., USA). Statistical comparisons were per-