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Short Communication

Insufficient Membrane Fusion in Dysferlin-Deficient Muscle Fibers after Heavy-Ion Irradiation

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ABSTRACT. **Recently, SJL/J mice have been used as an animal model in studies of dysferlinopathy, a spectrum of muscle diseases caused by defects in dysferlin protein. In this study we irradiated muscle fibers isolated from skeletal muscle of SJL/J mice with heavy-ion microbeam, and the ultrastructural changes were observed by electron microscopy. The plasma membrane of heavy-ion beam irradiated areas showed irregular protrusions and invaginations. Disruption of sarcomeric structures and the enhancement of autophagy were also observed. In addition, many vesicles of varying size and shape were seen to be accumulated just beneath the plasma membrane. This finding further supports the recent hypothesis that dysferlin functions as a membrane fusion protein in the wound healing system of plasma membrane, and that the defect in dysferlin causes insufficient membrane fusion resulting in accumulation of vesicles.**

Key words: heavy-ion microbeam/dysferlin/electron microscopy/vesicle accumulation/membrane repair

Introduction

Dysferlinopathy is a hereditary disease caused by mutation of the dysferlin gene (for review, see Han and Campbell, 2007) and consists of two major disorders, Miyoshi myopathy (Miyoshi *et al.*, 1986) and limb-girdle muscular dystrophy type 2B (LGMD2B) (Bashir *et al.*, 1998) and one minor disorder, distal anterior compartment myopathy (DAT) (Illa *et al.*, 2001). Dysferlin belongs to the ferlin-1-like proteins family, members of which have high homology to the *Caenorhabditis elegans* ferlin-1 (Han and Campbell, 2007). The members of the ferlin-1-like protein family contain multiple C2 domains, which bind with phospholipids in a Ca^{2+}-dependent manner (Davis *et al.*, 2002) and are considered to participate in membrane fusion by analogy with C2 domains of synaptotagmins (Bansal and Campbell, 2004). It has been reported that *C. elegans* ferlin-1 is essential for the fusion of large vesicles called membranous organelles (MOs) with the spermatid plasma membrane during maturation of spermatids to motile spermatozoa (Achanzar and Ward, 1997; Washington and Ward, 2006). Several studies have also reported that dysferlin is involved in a system for plasma membrane repair in response to Ca^{2+} influx (Han and Campbell, 2007). The *patch* hypothesis was proposed for membrane repair as follows: In normal muscle fiber, dysferlin is localized to the plasma membrane and cytoplasmic vesicles. When the membrane is damaged and then the Ca^{2+} level is increased at the damaged site, dysferlin triggers fusion of cytoplasmic vesicles each other and with plasma membrane and results in membrane patch. In the case of dysferlinopathy, it is postulated that the defect of dysferlin results in insufficient membrane fusion, which leads to Ca^{2+} influx and muscle degeneration. To test this *patch* hypothesis, it may be useful to induce microinjuries on the plasma membrane. Several techniques, including flushing through a syringe (Bansal *et al.*, 2003), laser irradiation (Bansal *et al.*, 2003; Han and Campbell, 2007),

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scalpel dragging (Lennon *et al.*, 2003) and glass bead wounding (Klinge *et al.*, 2007) have been employed for this purpose, and experiments using these methods have provided insight into the physiological function of dysferlin. However, no previous studies have demonstrated the ultrastructural alterations in the muscle wounded by the techniques listed above. Previously, we demonstrated that high-energy heavy-ion microbeam irradiation caused ultrastructural changes in the vicinity of the plasma membrane (Hino *et al.*, 2007). These ultrastructural changes indicated damage to the plasma membrane and increase of Ca^{2+} by the heavy-ion beam irradiation, and that heavy-ion beam can be employed in studying muscular dystrophy. SJL/J mice have a defect in the dysferlin gene (Bittner *et al.*, 1999; Vafiadaki *et al.*, 2001) and have been used as an animal model of dysferlinopathy. Here, we examined the ultrastructural changes of skeletal muscle fibres from SJL/J mice after heavy-ion beam irradiation to investigate the wound resealing system of plasma membrane.

Materials and Methods

All procedures were performed as described previously (Hino *et al.*, 2007). Briefly, single muscle fibers from the extensor digitorum longus (EDL) and flexor digitorum brevis (FDB) of 4- to 8-week-old female SJL/J and ICR mice were isolated by collagenase digestion. Isolated muscle fibers were plated on either 50–100 μm-thick CR-39 plastic ion-track detector (TNF-1, Fukuvi Chemical Industry, Fukui, Japan) or 120–170 μm-thick glass coverslips (Matsunami Glass, Osaka, Japan) depending on the experiment, and incubated at 37°C in 5% CO_2 for 1–4 days before irradiation experiments. The protocol used in this study was approved by the Animal Care and Experimentation Committee, Gunma University (No. 60108).

Cultured single fibers of skeletal muscles were irradiated with collimated heavy-ion microbeam at TIARA (Takasaki Ion Accelerator for Advanced Radiation Application) of the Japan Atomic Energy Agency (JAEA), for which the setup and irradiation procedures have been described previously (Funayama *et al.*, 2008b; Kobayashi *et al.*, 2004). Irradiated heavy-ions were ^{40}Ar (11.2 MeV/u) and ^{20}Ne (12.8 MeV/u) which were provided by the azimuthally-varying-field (AVF) cyclotron and extracted into the air through a 20-μm-diameter microaperture as reported previously (Funayama *et al.*, 2008b; Kobayashi *et al.*, 2004). Cells were irradiated at the edge using on-line microscope system. Each region was irradiated with 20 ion particles, and irradiated sites were visualized by etching the CR-39 film. The irradiated muscle fibers were fixed, processed and examined by an electron microscope (Hitachi model H-800B, Tokyo, Japan) as previously described (Hino *et al.*, 2007).

For measurement of the numbers of vesicles and vacuoles, electron micrographs were scanned (GT-X900; Seiko Epson Co., Tokyo, Japan) and the vesicles and vacuoles in the juxtamembrane regions, excluding the sarcomere, were counted. The measurement of the area was performed by Image J software (http://www.ncbi.nlm.nih.gov). Comparisons between groups were done using nonparametric Mann-Whitney test.

Results

We analyzed the alteration in the submembrane ultrastructure of the muscle fibers from the dysferlin-deficient SJL/J mice induced by heavy-ion microbeam irradiation. First, non-irradiated muscle fibers of SJL/J mice were observed (Fig. 1a). At the ages used (4 to 8 weeks old), there was no apparent difference between wild-type ICR mice (Hino *et al.*, 2007) and SJL/J mice (Fig. 1a) as determined by electron microscopy. A previous histological study also indicated that in SJL/J mice dystrophic changes appeared at about 60 days after birth and increased thereafter (Nemoto *et al.*, 2007). Muscle fibers fixed at 2 min after irradiation showed irregular protrusions and invaginations of the plasma membrane and disorganized basal lamina at the heavy-ion microbeam-irradiated regions (Fig. 1b). In the cytoplasm, an irregular distribution of microfilaments was found near the plasma membrane. These structural changes were also observed when muscle fibers from wild-type ICR mice were irradiated (Hino *et al.*, 2007). In addition to these changes, many vesicles of various sizes and shapes were accumulated just beneath the plasma membrane (Fig. 1b arrowheads). At 10 min after irradiation, massive protrusion of cytoplasm was observed (Fig. 1c). These protruded areas were occupied by vesicles (arrowheads) of varied size and morphology, and the myofibrillar structure disappeared in these areas (Fig. 1c). The accumulation of such vesicles observed in the muscle fibers of SJL/J mice (Fig. 1e) was slightly observed in those of wild-type ICR mice after irradiation (Fig. 1d). The accumulation of vesicles and vacuoles in irradiated SJL/J mice muscle fibers was also confirmed by quantitative analysis (Fig. 1f). In SJL/J mice, heavy-ion irradiation significantly induced the vesicles and vacuoles. The induction of vesicles and vacuoles accumulation was significantly higher in SJL/J mice than wild-type ICR mice.

At 2 min after irradiation, isolation membranes, which are precursor structures of autophagosomes (Mizushima *et al.*, 2002) and developing autophagosomes were observed in the cytoplasm of the irradiated area (Fig. 2a). A number of isolation membranes were not attached to mitochondria, suggesting that the non-selective autophagy took place. At 20 min after irradiation, autophagic vacuoles of various stages were observed (Fig. 2b). Frequency of the autophagosome formation did not appear to be depressed compared with the muscle fibers of wild-type mice (data not shown).

Discussion

Previously, we demonstrated that heavy-ion beam causes

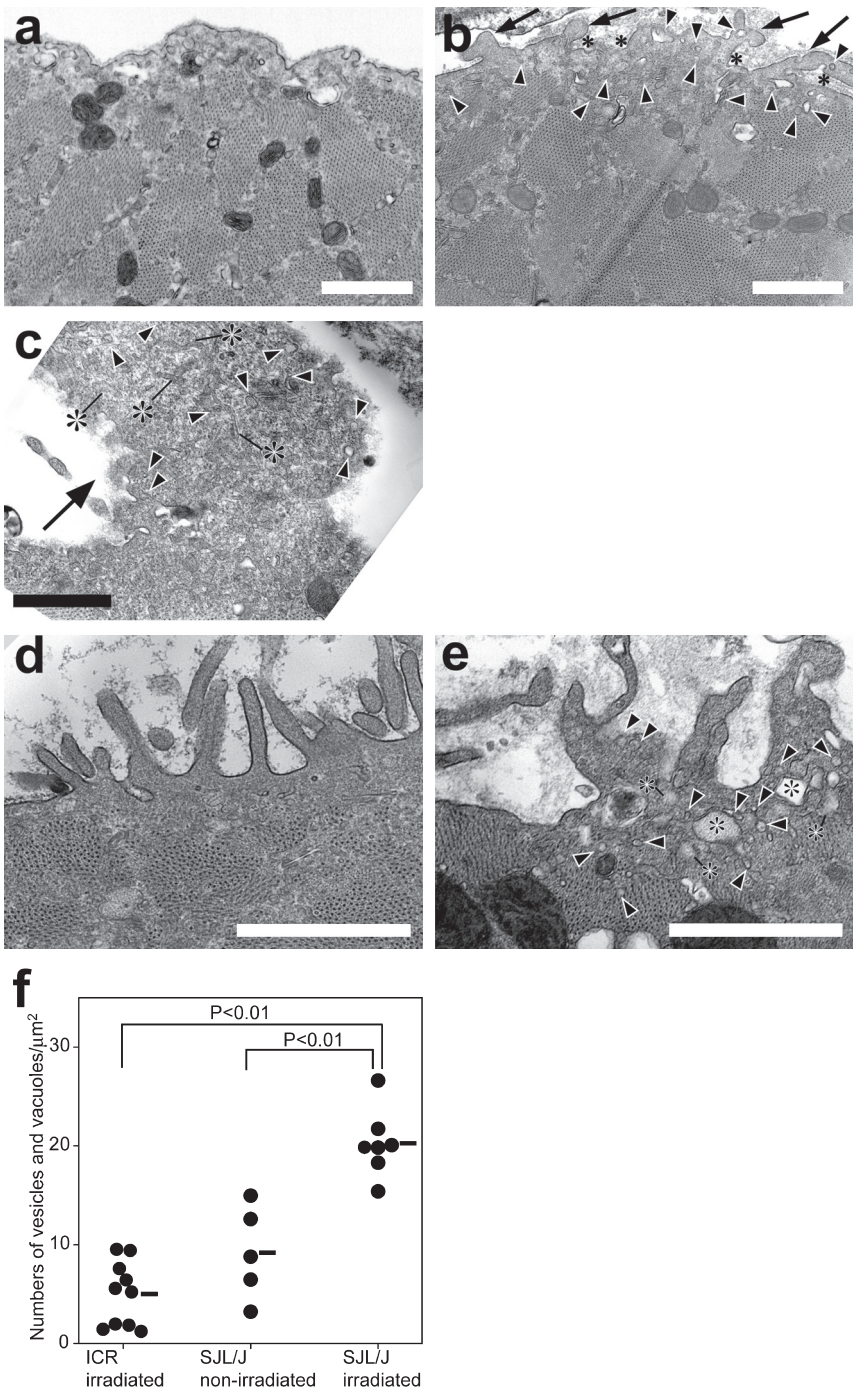

Fig. 1. Morphological changes of muscle fibers from SJL/J and ICR mice by heavy ion beam irradiation. (a) Non-irradiated region of muscle fiber of SJL/J mice. (b) Muscle fiber of SJL/J mice 2 min after ^{20}Ne ion irradiation. Irregular protrusions (arrows) and invaginations (asterisks) of plasma membrane are seen. Many vesicles vary in size and shape (arrowheads) are accumulated just beneath the plasma membrane. (c) Muscle fiber of SJL/J mice 10 min after ^{40}Ar ion irradiation. Massive protrusion (arrow) of cytoplasm containing many vesicles (arrowheads) and irregular endoplasmic reticulum-like organelles (asterisks) is shown. (d) Muscle fiber of ICR mice 20 min after ^{20}Ne ion irradiation. (e) Muscle fiber of SJL/J mice 20 min after ^{20}Ne ion irradiation. Many vesicles (arrowheads) and vacuoles (asterisks) are observed. Scale bar=1 μm. (f) Enhancement of vesicles and vacuoles accumulation in heavy-ion irradiated SJL/J mice muscle fibers. The numbers of vesicles and vacuoles per micrometer square of muscle fibers of ^{20}Ne ion irradiated ICR mice (n=10), non-irradiated SJL/J mice (n=5) and ^{20}Ne ion irradiated SJL/J mice (n=7) were plotted. Bars show the mean of each group. Values of p were calculated using Mann-Whitney test.

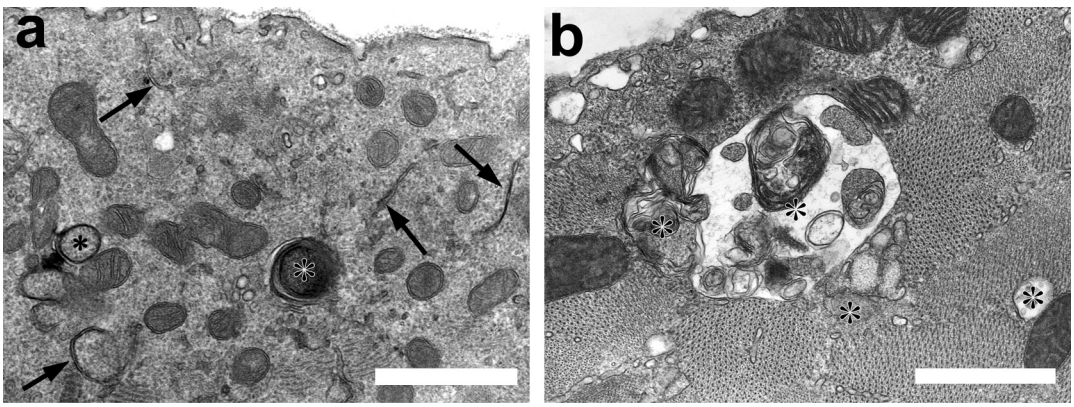

Fig. 2. Induction of autophagy by heavy-ion beam irradiation. Electron microscopy of the muscle fibers of SJL/J mice is shown. (a) Two min after ^{20}Ne ion irradiation. Isolation membranes (arrows) and developing autophagosomes (asterisks) are seen in the cytoplasm. (b) Twenty min after ^{20}Ne ion irradiation. Autophagic vacuoles of various stages (asterisks) are seen. Scale bar=1 μm.

ultrastructural lesion juxtamembrane area of isolated muscle fibers from skeletal muscles of wild-type mouse and suggested that this type of treatment can be used in research regarding muscular dystrophy (Hino *et al.*, 2007). Here we used muscle fibers isolated from dysferlin-deficient SJL/J mice and analyzed the ultrastructural changes after heavy-ion beam irradiation. The heavy-ion beam-induced changes in the muscle fibers of SJL/J mice observed in this study were as follows: i) irregular protrusions and invaginations of the plasma membrane; ii) disarrangement of the myofilaments; iii) enhanced autophagy; and iv) accumulation of many vesicles of various sizes and shapes just beneath the plasma membrane. Among these phenomena, i), ii) and iii) were also observed in irradiated muscle fibers from wild-type mice (Hino *et al.*, 2007). Irregular protrusions and invaginations of the plasma membrane might be caused by oxidation of lipids in the plasma membrane by ionization effect of heavy-ion beam. In muscle fibers from SJL/J, massive protrusion of cytoplasm was observed (Fig. 1c). This may have been caused by insufficient membrane repair of SJL/J mice. Autophagy is one of the major pathways for degradation of intracellular macromolecules and organelles (Mizushima *et al.*, 2002). We conjectured that autophagy is the principal mechanism for removal of the damaged cellular components, such as cell membrane, sarcomeric structure and mitochondria, after irradiation. Enhancement of autophagy by irradiation was observed in muscle fibers both from SJL/J and wild-type mice. These observations indicated that the induction of autophagy by heavy-ion beam is independent of dysferlin.

The most striking characteristic of heavy-ion irradiated muscle fibers of SJL/J mice in comparison with wild-type control was the accumulation of vesicles just beneath the plasma membrane. These vesicles varied in both size and morphology, and could not be identified as to their origins. Accumulation of such vesicles just beneath the plasma membrane has been observed in the skeletal muscles of patients with dysferlinopathy (Cenacchi *et al.*, 2005), and in dysferlin-deficient mice at 20 weeks (Ho *et al.*, 2004) and at one year old (Bansal *et al.*, 2003). For irradiation, we used mice up to 8 weeks old, which did not show any marked dystrophic changes, especially the significant vesicle accumulation, before irradiation (Fig. 1a and 1f). These vesicles were appreciably accumulated as early as 2 min after irradiation in SJL/J mice. It is interesting to note that dysferlin is dispensable for this event. Whether these vesicles were transported from non-irradiated area or constructed at irradiated area is unclear. Taken together, our results suggested that the beginning of the membrane repair is the accumulation of vesicles at wounded area in response to the membrane damage in the dysferlin independent manner. The dysferlin-dependent patch process then initiates the membrane repair in wild type muscle.

Recently, laser-assisted microsurgery has been used in the field of cell biology. What is the advantage of heavy-ion microbeam over a laser beam for microsurgery of biological objects? The effects of laser irradiation on biological samples are heating action and subsequent hydrodynamic effects such as shockwave and collapse of cavitation bubbles (Vogel *et al.*, 2005). These laser-induced effects spread over the focus with low target selectivity. (Funayama *et al.*, 2008a) On the other hand, the main effect of the heavy-ion irradiation is the ionization of molecules along condensed ion tracks (Kiefer and Straaten, 1986) with high target selectivity (Funayama *et al.*, 2008a). Smaller lesions can be made using a heavy ion beam than with laser irradiation. Therefore, heavy-ion beam irradiation is suitable for ultrastructural studies, such as electron microscopy. However, the irradiation systems of heavy-ion beam are restricted as compared with laser-assisted microsurgery, which is easily available and inexpensive. We should complement these two methods by using properly according

to the situation.

Recently, it has been reported that mitsugumin 53 (MG53), a muscle-specific tripartite motif family protein (TRIM72), plays a role in plasma membrane-repair machinery (Cai *et al.*, 2008). This protein may have some role in vesicles/vacuoles accumulation in heavy-ion irradiated muscle fibers of SJL/J mice.

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