Heavy Ion Microbeam Irradiation Induces Ultrastructural Changes in Isolated Single Fibers of Skeletal Muscle

Mizuki Hino1,2, Seiichi Wada1,3,4,5, Yuki Tajika1, Yoshihiro Morimura1, Nobuyuki Hamada3,4, Tomoo Funayama1, Tetsuya Sakashita1, Takehiko Kakizaki1,5, Yasuhiko Kobayashi2,3,4, and Hiroshi Yorifuji1∗

1Department of Neuromuscular and Developmental Anatomy, Division of Bioregulatory Medicine, Gunma University Graduate School of Medicine, Maebashi, Gunma, 371-8511, Japan, 2The 21st Century Center of Excellence Program for Biomedical Research Using Accelerator Technology, Maebashi, Gunma, 371-8511, Japan, 3Microbeam Radiation Biology Group, Radiation-applied Biology Division, Quantum Beam Science Directorate, Japan Atomic Energy Agency, Takasaki, Gunma, 370-1292, Japan, 4Department of Quantum Biology, Division of Bioregulatory Medicine, Gunma University Graduate School of Medicine, Maebashi, Gunma, 371-8511, Japan, 5Present address: Laboratory Veterinary Radiology, School of Veterinary Medicine and Animal Science, Kitasato University, Higashi 23 bancho 35-1, Towada, Aomori, 034-8628, Japan

ABSTRACT. The effects of heavy ion microbeams on muscle fibers isolated from mouse skeletal muscles were examined by electron microscopy. The plasma membranes of heavy ion beam-irradiated areas of muscle fibers showed irregular protrusions and invaginations. In the cytoplasm, an irregular distribution of microfilaments was found near the plasma membrane. Sarcoplasmic reticula in the irradiated regions showed a distended appearance with flocculent material within the lumen. These changes were seen as early as 2 min after irradiation, and persisted until as late as 22 min after irradiation. Many autophagic vacuoles could be seen at 7 min after irradiation. At 22 min, the vacuoles became more prominent and showed more variety. These observations suggest that heavy ion beam irradiation causes disruption of the cellular architecture and the autophagy is involved in removal of this disruption.

Key words: heavy ion microbeam/membrane aberration/calcium ion influx/autophagy

Introduction

Muscular dystrophy is a heterogeneous group of hereditary diseases characterized by progressive muscle weakness and wasting. Recently, molecular genetics studies have elucidated a variety of affected genes and their products in muscular dystrophy (Kanagawa and Toda, 2006). Many of these proteins are localized to the basal lamina, plasma membrane, or the underlying membrane skeleton, and have been implicated in plasma membrane integrity. In this type of muscular dystrophy, microinjury of the plasma membrane and the resultant Ca2+ influx are thought to result in muscle degeneration (Gissel, 2005). However, no model system for inducing microinjury of the skeletal muscle plasma membrane and observing its resultant turnover has yet to be reported.

Heavy ion beams are high linear energy transfer (LET) ionizing radiation and produce dense ionization (Blakely and Kronenberg, 1998). Heavy ions are more effective in inducing various biological endpoints than low-LET photons, such as X- or γ-rays, and heavy ion therapy of malignant tumors is currently in progress (Jäkel et al., 2003). It is also important to evaluate the effects of cosmic ray exposure in space activity, especially during extravehicular activity (Ohnishi and Ohnishi, 2004). However, in contrast to the large number of reports regarding alterations of physiological function (early fatigue, contracture, prolongation of relaxation time, and decreased contraction amplitude for heavy loads), histological changes, such as myofibrillosis and necrosis (Khan, 1974), edema (Nömayr et al., 2001), and release of amino acids, especially alanine and glutamine (Schwenen et al., 1989; Holecek et al., 2002) associated with exposure to X- or γ-rays, there have been no
previous reports describing the effects of heavy ion beams on skeletal muscle. To determine whether the muscle cell plasma membrane suffers microinjury and undergoes resultant turnover, we carried out target irradiation of isolated skeletal muscle fibers with heavy ion microbeams. This approach has advantages over conventional “broad-field” irradiation, which involves the stochastic dispersal of heavy ion particles, in that it allows irradiation of individual cells in a specific region with a precise number of particles (Funayama et al., 2005). Muscle fibers have been irradiated at the edge using Ar- or Ne-microbeams. Here, we report disturbance of the architecture of plasma membrane and myofilaments, and the enhancement of autophagy after irradiation.

Materials and Methods

Cell culture

Single muscle fibers from the extensor digitorum longus (EDL) and flexor digitorum brevis (FDB) of 4- to 8-week-old female ICR mice were isolated and cultured according to the protocols of Partridge (Partridge, 1997), except that collagenase type I (lot M4C7120; Worthington Biochemical Co., Lakewood, NJ) instead of collagenase (from Sigma, St. Louis, MO) was used for digestion, and 20% fetal bovine serum in MEM containing 1.25 ng/ml basic fibroblast growth factor (bFGF) instead of 10% horse serum. Isolated muscle fibers were plated on either 100-μm-thick CR-39 plastic ion-track detector (TNF-1; Fukuvi Chemical Industry, Fukui, Japan), or glass coverslips depending on the experiment, and incubated at 37°C in 5% CO₂ for 1–4 days before irradiation experiments. The protocol followed in this study was approved by the Animal Care and Experimentation Committee, Gunma University, Showa Campus (No. 60108).

Irradiation

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) (Kobayashi et al., 2004). Irradiated heavy ions were 40Ar (11.2 MeV/u) and 28Ne (12.8 MeV/u), which were provided by the azimuthally varying field (AVF) cyclotron and extracted into the air through a microaperture 20 μm in diameter as reported previously (Kobayashi et al., 2004). Cells were irradiated at the edge using an on-line microscope system, designed to target specific regions of biological samples with heavy ion particles. In each region, 10 ion particles were irradiated. Irradiated points were visualized by etching the CR-39 film with KOH-ethanol solution (Wada et al., 2002; Funayama et al., 2005).

Electron microscopy

The irradiated muscle fibers were fixed at various time points after irradiation. The experimentally shortest time possible between the end of irradiation and fixation was 2 min. The irradiated and non-irradiated control specimens were fixed with a fixative containing 2.5% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.3, with or without 0.2% tannic acid. After washing with 10% sucrose, 0.1 M sodium cacodylate, pH 7.3, they were post-fixed with 1% OsO₄, 0.1 M sodium cacodylate, pH 7.3. They were then dehydrated by passage through a graded ethanol series and embedded in Epon 812 (TAAB Laboratories Equipment Ltd., Berkshire, UK). After etching of ion pits, the CR-39 film was trimmed for electron microscopy. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined by electron microscopy (model H-800B; Hitachi, Tokyo, Japan).

Results

In this study, we analyzed the local response of the muscle fibers to heavy ion microbeam irradiation. Muscle fibers fixed at 2 min (the shortest time point after the end of irradiation) showed irregular protrusions and invaginations of the plasma membrane and fragmented basal lamina in the heavy ion microbeam-irradiated regions (Fig. 1b). In the non-irradiated area (Fig. 1a), small invaginations called caveolae were seen. However, the extent of the invagination was smaller than that of the irradiated area. In the cytoplasm, an irregular distribution of microfilaments was found near the plasma membrane. Thick filaments were scattered irregularly around the periphery of the groups of thin filaments (Fig. 1b). Some thick filaments were found near the Z-disks. These observations indicated the local irregular contraction of sarcomeres, whereas myofilaments showed well-arranged topography in the non-irradiated area (Fig. 1a). Sarcomplasmic reticula near the irradiated regions showed a distended appearance with flocculent material within the lumen (Fig. 1b). The irregularity of myofilaments and distended sarcoplasmic reticula remained at 22 min (data not shown).

Many autophagic vacuoles could be seen at 7 min after irradiation (Fig. 2a), although at 2 min no increase in number of autophagic vacuoles as compared with the non-irradiated controls could be observed. Slender and elongated membranous structures, probably isolation membranes, which are the intermediates of the autophagosomes (Mizushima et al., 2002), were also found around some mitochondria (Fig. 2a). In some cases, mitochondria or part of the cytoplasm were concentrically encircled by autophagic vacuoles that seemed to be immature as the contents retained the original structures. At 22 min, the vacuoles became more prominent and showed more variety (Fig. 2b). Advanced stages of the autophagic vacuoles with well-digested contents could be seen.
Fig. 1. Morphological changes in the muscle fiber following heavy ion beam irradiation. Electron micrographs of the $^{40}\text{Ar}$-irradiated muscle fiber fixed 2 min after the end of irradiation are shown. (a) Non-irradiated region. Caveolae (arrows) were seen on the plasma membrane. Myofilaments showed a well-arranged topography. Sarcoplasmic reticula (SR) appeared normal. Scale bar, 1 µm. (b) Irradiated area of the same cell as in Fig. 1a. Irregular protrusions (arrows) and invaginations (*) of the plasma membrane were seen. These invaginations were confirmed to be continuous with the extracellular space by examination of serial sections. Thick filaments (arrowheads) were scattered irregularly around the periphery of the groups of thin filaments. Distended sarcoplasmic reticula (SR) were also seen. m, mitochondria. Scale bar, 1 µm.

Fig. 2. Induction of autophagic vacuoles by heavy ion beam irradiation. Electron micrographs of the $^{20}\text{Ne}$-irradiated muscle fibers are shown. (a) Seven minutes after irradiation. Some mitochondria were surrounded by isolation membranes (arrows). Scale bar, 1 µm. (b) Twenty-two minutes after irradiation. Advanced stages of the autophagic vacuoles were seen (arrows). N, nucleus. Scale bar, 1 µm.
Discussion

The heavy ion-induced changes in the isolated skeletal muscle fibers observed in the present study were as follows: i) irregular protrusions and invaginations of the plasma membrane, ii) disarrangement of the myofilaments, iii) distended sarcoplasmic reticula, and iv) enhanced autophagy. The differences in the effects on muscle fibers between the two types of ions used ($^{40}$Ar and $^{20}$Ne with LET of 1260 and 375 keV/$\mu$m) seemed to be quantitative rather than qualitative, i.e., Ar ions had a more intense and massive influence than Ne ions.

Irregular protrusions and invaginations of the plasma membrane are thought to be caused by the changes in the fluidity of the plasma membrane. In vitro studies indicated that heavy ion beams induce dimerization/shortening of membrane lipid by oxidation and alter the membrane fluidity (Schön et al., 1994; Choudhary et al., 1998; Ziegler and Wessels, 1998).

Disarrangement of the myofilaments and distended sarcoplasmic reticula may reflect local transient leakage of Ca ions into the cytoplasm. There are three possible pathways of calcium influx into the cytoplasm of muscle fibers: from the sarcoplasmic reticulum, the mitochondria, and from the extracellular environment. The observation that heavy ion irradiation may form holes in liposomes (Koniarék et al., 2004) raises the possibility that calcium ions enter the cytoplasm through holes induced by the heavy ion beam in the lipid bilayer of the plasma membrane and/or sarcoplasmic reticula. However, in our preliminary experiments, permeabilization of the plasma membrane by the heavy ion beam was not observed as judged by the invasion of fluorescent dextran (MW=10,000) and Evans blue (MW=961) (data not shown). We consider that the contribution of such holes to calcium leakage, especially on the plasma membrane, is small. On the other hand, ion channels have been shown to mediate entry of calcium into the cytoplasm under physiological conditions (Gailly, 2002). Of these ion channels, ryanodine receptor 1, a major Ca ion channel located on sarcoplasmic reticulum membrane in skeletal muscle, has been reported to be opened reversibly by nitric oxide (NO) via S-nitrosylation of Cysteine-3635 (Cheong et al., 2005; Hidalgo et al., 2005). It is well known that ionizing radiation stimulates NO formation (Mikkelsen and Wardman, 2003). With regard to heavy ion irradiation, it has been reported that NO is involved in bystander effects on cell growth stimulation induced by $^{12}$C irradiation in neoplastic human salivary gland cells (Shao et al., 2003). The local transient leakage of calcium ions caused by irradiation with a heavy ion beam as observed in the present study may result from the production of NO species followed by S-nitrosylation of ryanodine receptor 1.

Although there has been a great deal of recent progress in elucidation of the molecular mechanisms of autophagy, the detailed overview remains to be clarified as there are too many steps and too many factors involved (Mizushima et al., 2002). The pattern and time course of maturation of autophagic vacuoles induced by heavy ion irradiation coincided well with those induced by starvation (Mizushima et al., 2001). This observation suggested that autophagy induced by heavy ion irradiation by starvation may share the same machinery. Although autophagy is considered a non-selective pathway, selective autophagy in which mitochondria are selectively degraded, has been designated “mitophagy” (Jin, 2006). In the present study, the majority of autophagic vacuoles contained mitochondria, but some vacuoles did not. Here, we could not determine whether the heavy ion-induced autophagy was non-selective or selective mitophagy. However, we considered that autophagy is the principal mechanism for removal of the damaged cellular components, such as cell membrane, sarcomeric structures, and mitochondria. In general, the autophagic vacuoles are not remarkable in normal muscle. On the other hand, they are present in large numbers in muscular diseases, such as distal myopathy with rimmed vacuoles (DMRV) (Nonaka et al., 1981), acid maltase deficiency (Amato, 2000), and Danon disease (Danon et al., 1981), and thus represent a useful index for pathological diagnosis. These diseases, collectively known as autophagic vacuolar myopathy, are assumed to be caused by the deficiency of lysosomal enzymes within the vacuoles and the lysosomal membrane structural protein (Nishino, 2006). Irradiation of skeletal muscles with heavy ion beams would contribute to elucidation of the mechanisms of autophagic vacuole accumulation in these diseases.

Along with the nerves, skeletal muscle is the tissue that suffers the effects of mitochondrial disorders most intensely (DiMauro, 2006). As mitochondria possess their own DNA, proliferate, and are the sites that produce reactive oxygen species (ROS), it is considered that mitochondria are important targets of ionizing radiation in the cell (Somosy, 2000). There have been no previous studies regarding mitochondrial DNA (mtDNA) damage induced by heavy ion beams. On the other hand, X- and $\gamma$-ray-induced mtDNA damage have been documented extensively (Murphy et al., 2005). As heavy ion beams evoke denser double-strand breaks of nuclear DNA than X- or $\gamma$-rays (Fakir et al., 2006), heavy ion beams are highly likely to cause more severe damage to mtDNA. In addition to the effects on DNA, X-rays evoke ROS-induced ROS formation via mitochondrial permeability transition (MPT) (Leach et al., 2001). While limited MPT was suggested to result in autophagy (Rodriguez-Enriquez et al., 2004), heavy ion-induced autophagy of mitochondria may be mediated through this pathway.

Previous studies of cellular radioresponse focused on intranuclear processes, especially nuclear DNA damage-induced cascades. However, it has recently been accepted that the quality control of cytoplasmic components, such as mitochondria, cytoskeleton, endoplasmic reticulum, and plasma membrane, is important for the radioresponses of...
Effects of Heavy Ion Irradiation on Muscle Cells

the cell (Somosy, 2000). In the present study, we showed that dynamic changes are generated in the cytoplasm of skeletal muscle cells by heavy ion irradiation.

Microbeam irradiation by heavy ion beams is not specific to the plasma membrane. Although irradiation was aimed at the edge of the single fibers of the skeletal muscle, the area of irradiation included the cytoplasm beneath the plasma membrane. This was because scattering of the beams is inevitable, although the beams were collimated as precisely as possible. Damage by the beams may not be restricted to the plasma membrane, but also to the structures inside the cell, such as the mitochondria, myofibrils, and sarcoplasmic reticula. We do not consider the changes in the arrangement of myofibrils to be the result of the direct effects of heavy ion irradiation. As discussed above, these changes may be caused by the increase in intracellular calcium level.

Irradiation of isolated skeletal muscle fibers by heavy ion beams can be used as a limited model system in which to study muscular dystrophy. Turnover of the damaged plasma membrane may also be different between this heavy ion beam irradiation model and muscular dystrophy as discussed above. No useful experimental systems regarding the turnover of the damaged plasma membrane in the skeletal muscle cells by heavy ion irradiation can be used as a limited model system in which to study muscular dystrophy. Turnover of the damaged plasma membrane may also be different between our experimental conditions. The rise in intracellular calcium may also be different between this heavy ion beam irradiation model and muscular dystrophy as discussed above. No useful experimental systems regarding the turnover of the plasma membrane of the skeletal muscle are presently available. Irradiation by heavy ion beams may thus be useful to study the damaged membranes in the skeletal muscle.

Acknowledgments. We thank Dr. Shin’ichi Takeda and Dr. Yuko Miyagoe-Suzuki at the National Center of Neurology and Psychiatry for instruction regarding the methods for isolation of single skeletal muscle fibers. We thank the operators of TIARA of JAEA for technical assistance. This work was supported by a Grant-in-Aid for the 21st Century Center of Excellence Program for Biomedical Research Using Accelerator Technology from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This work was also supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to H.Y.) and a Research Grant (17A-10) for Nervous and Mental Disorders from the Ministry of Health, Labor, and Welfare of Japan (to H.Y.).

References


(Received for publication, December 22, 2006 and accepted March 10, 2007)