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Research paper

APOBEC2 negatively regulates myoblast differentiation in muscle regeneration

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ABSTRACT

Recently we found that the deficiency of APOBEC2, a member of apoB mRNA editing enzyme, catalytic polypeptide-like family, leads to a diminished muscle mass and increased myofiber with centrally-located nuclei known as dystrophic phenotypes. APOBEC2 expression is predominant in skeletal and cardiac muscles and elevated exclusively at the early-differentiation phase of wild-type (WT) myoblast cultures; however the physiological significance is still un-known. Here we show that APOBEC2 is a key negative regulator of myoblast differentiation in muscle regeneration. APOBEC2-knockout (A2KO) mice myoblast cultures displayed a normal morphology of primary myotubes along with earlier increase in fusion index and higher expression levels of myosin heavy chain (MyHC), myogenin and its cooperating factor MEF2C than WT myoblasts. Similar response was observable in APOBEC2-knockdown cultures of WT myoblasts that were transfected with the specific siRNA at the differentiation phase (not proliferation phase). Importantly, cardiotoxin-injured A2KO gastrocnemius muscle provided *in vivo* evidence by showing larger up-regulation of neonatal MyHC and myogenin and hence earlier regeneration of myofiber structures with diminished cross-sectional areas and minimal Feret diameters. Therefore, the findings highlight a promising role for APOBEC2 in normal progression of regenerative myogenesis at the early-differentiation phase upon muscle injury.

1. Introduction

Satellite cells are resident myogenic stem cells and are normally found in a quiescent state in adult animals (Mauro, 1961; Snow, 1997). In response to muscle injury, the cells are quickly activated in a hepatocyte growth factor/NO radical-dependent manner; once activated, they proliferate into myoblasts, differentiate, and fuse to damaged myofibers (or form new fibers) (Tatsumi et al., 1998; Anderson, 2000; Hawke and Garry, 2001; Tatsumi and Allen, 2008; Tatsumi, 2010). These sequential events are highly regulated by a variety of intracellular and extracellular molecules including transcription factors, cell-membrane and nuclear receptors, growth factors, cytokines, and hormones (Bentzinger et al., 2010; Turner and Badylak, 2012). The mechanisms that coordinate these various molecules are not yet fully understood, particularly for the fascinating and dramatic steps of myoblast differentiation and fusion in regenerative myogenesis.

The family of myogenic regulatory factor (MRF) proteins, also called basic helix-loop-helix (bHLH) transcription factors, includes four MyoD, Myf5, myogenin, and MRF4; together they play crucial roles in myoblast differentiation and fusion. MyoD expression peaks in the mid-G1 phase of the cell cycle; MyoD expression permits myoblast differentiation and ultimately leads to cell-cycle arrest, while Myf5 expression begins shortly after MyoD expression begins and helps determine the cell fate toward the myogenic lineage (Ishibashi et al., 2005; De Falco and De Luca, 2006; Gayraud-

Abbreviations: APOBEC, apoB mRNA editing enzyme catalytic polypeptide-like; CTX, cardiotoxin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HS, horse serum; MEF2C, myocyte enhancer factor 2C; MyHC, myosin heavy chain.

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Morel et al., 2007; Ustanina et al., 2007). Importantly, myogenin is up-regulated at the early-differentiation phase and regulates the fusion of myoblasts into myotubes (immature myofibers) that is followed by MRF4-dependent myotube maturation (Venuti et al., 1995; Zhang et al., 1995; Zanou and Gailly, 2013).

APOBEC2 is a member of the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like family of proteins having zinc-dependent cytidine deaminase activity (Liao et al., 1999; Navaratnam and Sarwar, 2006; Conticello, 2008). Recently, we found that a deficiency of APOBEC2 leads to a diminished mouse muscle mass and increased myofiber with centrally-located nuclei known as dystrophic phenotypes (Sato et al., 2009, 2010). Experiments also demonstrated that APOBEC2 expression is predominant in skeletal and cardiac muscles and in cultured wild-type (WT) myoblasts, its expression rises exclusively in the early-differentiation phase along with its intracellular translocation from the nucleus to the cytoplasm of myoblasts. Additional information was provided by a Zebrafish study to demonstrate that APOBEC2 protein is localized at the Z-line region of sarcomeres along the myofibril and that APOBEC2 knockdown leads to a myopathic phenotype (Etard et al., 2010). These reports encourage a model in which APOBEC2 mediates differentiation/fusion of myoblasts responsible for the formation of myotubes during muscle regeneration from injury, even though there is a limited number of references on APOBEC2 biology, including some recent studies to show that APOBEC2 may participate in the left-right axis determination in *Xenopus* embryogenesis (Vonica et al., 2011) and retina regeneration independent on cytidine deaminase activity in zebrafish (Powell et al., 2012, 2014). Thus it is important to clarify the biological and physiological roles of APOBEC2 in muscle regeneration.

The present study was designed to examine the effect of APOBEC2 knockout in primary satellite cell cultures and *in vivo* in a model of muscle injury. Results clearly showed that APOBEC2 deficiency accelerates the formation of primary myotubes in culture and increases the up-regulation of differentiation markers including myosin heavy chain (MyHC), myogenin, and myocyte enhancer factor 2C (MEF2C) which cooperates with myogenin. The enhanced differentiation and fusion observed in culture after APOBEC2 knockout may explain the accelerated restoration of myofiber structures in regeneration of muscle after *in vivo* injury. Therefore, APOBEC2 functions as a negative regulator in regeneration of wild-type muscle, and its role centers on modulating processes of myoblast differentiation and fusion.

2. Materials and methods

2.1. Animal care and use

APOBEC2-deficient mice (A2KOs; C57BL/6 as the background strain) were generated by Dr. Neuberger (Medical Research Council Laboratory of Molecular Biology, United Kingdom) and bred in our laboratory (Mikl et al., 2005). Inbred C57BL/6 mice were used as wild-type (WT) controls. All animal experiments were conducted in strict accordance with the Guidelines for Proper Conduct of Animal Experiments published by the Science Council of Japan and ethics approvals from the Kyushu University Institutional Review Board (approval nos. 20–12, 23–62, A22–218, A24–075, A26–078, and A28–090).

2.2. Satellite cell isolation and primary culture

Satellite cells were isolated from body muscles of 4-month-old WT and A2KO mice according to Jackson et al. (1999) with a slight modification and used for all culture experiments (except for APOBEC2-knockdown experiments shown in Fig. 3). Briefly, muscle was treated 0.2% collagenase type II (LS0041 from Worthington, Lakewood, NJ) for 45 min followed by 0.1% trypsin (15400, Thermo Fisher Scientific, Waltham, MA) for 45 min at 37 °C. Cells were collected by centrifugation and plated on a non-coated dish for 2 h. Non-adherent cells were collected and plated on plastic dishes and 8-well chamber slides (for measuring fusion index) coated with Matrigel (356234, BD Bioscience, Bedford, MA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS; Invitrogen, Grand Island, NY), 1% antibiotic-antimycotic mixture (15240-062, Thermo Fisher Scientific) and 0.5% gentamicin (15710-64) (proliferation medium) until about 70% confluence. Subsequently, cell differentiation was induced by serum starvation by altering the culture medium to DMEM containing 5% horse serum (HS; 16050-122, Invitrogen), 1% antibiotic-antimycotic and 0.5% gentamicin (differentiation medium). Myotube formation was evaluated by the measurement of fusion indices (the number of myonuclei as a percentage of total nuclei, visualized with 4′,6-diamidino-2-phenylindole, DAPI) at five random fields for each well (at least 5000 nuclei counted per well, $n = 3$ wells per group). Companion satellite-cell cultures, prepared at the same time, were immunostained for the presence of Pax7 at 48 h post-plating, using a monoclonal anti-Pax7 antibody (clone PAX7, Developmental Studies Hybridoma Bank, Iowa City, IA) and Alexa Fluor 594-labeled anti-mouse IgG secondary antibody (1:500 dilution; A11005 of Thermo Fisher Scientific), in order to determine the percentage of myogenic cells present; cultures with less than 95% Pax7-positive cells were not used for experiments.

2.3. Muscle injury

Mice were anesthetized by intraperitoneal injection (*ip*) with a mixture of agents (0.3 μg medetomidine hydrochloride, 4 μg midazolam, and 5 μg butorphanol tartrate per g body weight) and then had an intramuscular injection of 50 μl of 10 μM cardiotoxin (CTX; the peptidic myotoxin isolated from venom of the *Naja mossambica mossambica*; Sigma-Aldrich, St. Louis, MO) into each medial and lateral head of gastrocnemius muscle as optimized previously (Sakaguchi et al., 2014). CTX-injected muscles were dissected at day 0 (before injection), or 4, 7, 14, and 21 days after injection and frozen in isopentane (26405-65, Nacalai Tesque, Kyoto, Japan) cooled with liquid nitrogen. Muscle cryosections (mid-belly portions, about 7-μm thickness) were stained with hematoxylin (8650; Sakurai Finetek Japan, Tokyo, Japan) and eosin (8659; Sakurai Finetek Japan) and dehydrated with ethanol. Myofiber cross-sectional area and minimal Feret diameter (at least 800 fibers per group, $n = 3$) of muscles from WT and A2KO mice were measured using ImageJ 1.34 s software (originally developed by Dr. Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

2.4. Immunohistochemistry

Primary cultures of satellite cells were fixed with 4% paraformaldehyde (09154-85, Nacalai Tesque) and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS). Subsequently, cells were blocked with 3% bovine serum albumin (BSA; A8022, Sigma-Aldrich) in 0.1% polyethylene sorbitan monolaurate (Tween 20)-Tris buffered saline (TTBS) at room temperature for 1 h before incubation with monoclonal anti-pan MyHC antibody (1:500 dilution; clone MF20, IC4470F from R&D Systems, Minneapolis, MN) and polyclonal anti-MyoD antibody (1:200 dilution; sc-760 from Santa Cruz Biotechnology, Santa Cruz, CA) and with Alexa Fluor 594-labeled anti-mouse secondary antibody (1:500 dilution) and Alexa Fluor 488 anti-rabbit secondary antibody (1:500 dilution; A-21441, Invitrogen), respectively. Cells were counter-stained with