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Degenerative muscle fiber accelerates adipogenesis of intramuscular cells via RhoA signaling pathway

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ABSTRACT

In some pathological conditions such as Duchenne muscular dystrophy, it has been known that a fatty infiltration in skeletal muscle is often observed and that is also one of primary factors to induce marked decline of muscular strength. However, the mechanism of fatty infiltration, cellular origin of accumulated adipocytes and its significance are not fully understood. The fact that persistent degenerative muscle fibers are present on dystrophic muscle leads us to hypothesize that muscle fiber condition affects fatty infiltration in skeletal muscle. We employed a single fiber culture system to determine whether fiber condition affects an appearance of adipocytes on the fibers. Artificially hypercontracted muscle fibers (HCF), generated from isolated intact fibers (IF) of rat *extensor digitrum longus* muscle, were maintained as non-adherent cultures for 5–7 days. Interestingly, there appeared to be considerable numbers of mature adipocytes on HCF, whereas no adipocytes were seen on IF, indicating that cells on HCF spontaneously differentiated into mature adipocytes. Activation of RhoA signaling by the addition of thrombin decreased the number of adipocytes on HCF in a dose-dependent manner, whereas the number of MyoD-positive myoblasts increased. In contrast, Y-27632, a specific inhibitor of Rho kinases (ROCK), induced adipogenic differentiation of cells derived from IF. In addition, administration of Y-27632 into mouse regenerating muscle resulted in fat accumulation in the muscle. Taken together, the present studies clearly demonstrated that muscle fiber condition affects fat accumulation in skeletal muscle and that is possibly mediated by the RhoA signaling pathway.

1. Introduction

In adult skeletal muscle, there are several types of stem cells such as satellite cell, muscle side-population (SP) cells and muscle-derived stem cells (MDSCs), and these intramuscular stem cells are able to differentiate into adipogenic and osteogenic cells in addition to myogenic cells when they are cultured in each optimal conditions (Lee et al., 2000; Asakura et al., 2001, 2002; Wada et al., 2002; Uezumi et al., 2006).

Fat accumulation in skeletal muscle is often seen in patients with X-chromosome-linked recessive muscular dystrophy such as Duchenne muscular dystrophy (DMD), and is one of the primary factors leading to marked decline of muscular strength (Tyler, 2003). Fragility of muscle fibers caused by loss of dystrophin induces rapid influx of Ca^{2+} into skeletal muscle fiber, resulting in hypercontraction of muscle fibers and activation of proteases such as Calpain (Carpenter and Karpati, 1979). Moreover, repeated degeneration and regeneration in dystrophic muscle finally results in loss of muscle fibers, and adipocytes fill up in the area where muscle fibers are lost (Cullen and Mastaglia, 1980). Similarly, fat accumulation in skeletal muscle is also observed in atrophic muscle of elderly people and experimentally denervated-muscle (Dulor et al., 1998; Song et al., 2004).

In general, maintenance and fate specification of embryonic and somatic stem cells are affected by components of their surrounding microenvironments such as soluble factors, neighboring cells and extracellular matrices (ECM), and particular microenvironments are necessary for each stem cell type to be specified appropriately (Schofield, 1978). In *Drosophila*, for example, Decapentaplegic (Dpp), a BMP2/4 homolog, is required to maintain germline stem cells in the ovary and regulates their division, and an anchoring germline stem cell to a neighboring cap cells in the ovary mediated by DE-cadherin is important for stem cell maintenance and function (Song et al., 2002; Xie and Spradling, 1998). In addition, substrate elasticity of ECM also affects fate specification of human mesenchymal stem cells (Engler et al., 2006). Csete et al. (2001) demonstrated that oxidative stress induces adipogenic differentiation of muscle fiber associating cells using single muscle fiber culture. More recently, furthermore, Brack et al. (2007) demonstrated that aged microenvironment induces a conversion of satellite cells from myogenic to fibrogenic lineage, and this conversion is mediated by

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modification of Wnt signaling. Taken together, these results clearly indicate that microenvironment in skeletal muscle affects lineage specification of intramuscular cells.

RhoA is a member of the small GTPase Rho family including Rho, Rac, and Cdc42, and it is widely known that RhoA is associated with organization of the cytoskeleton (Hall, 1998; Ridley and Hall, 1992; Ridley et al., 1992). The RhoA signaling pathway is regulated via cell surface receptors such as G-protein coupled receptors (GPCRs), and is activated by the interaction between the receptors and ECM and/or soluble factors (Ren et al., 1999). In myogenic cell lines such as C2C12 and L6 myoblasts, the RhoA signaling pathway participates in myotube formation (Carnac et al., 1998; Castellani et al., 2006; Charrasse et al., 2006; Reuveny et al., 2004; Wei et al., 1998). These observations suggest that RhoA functions in myogenic cells. However, whether RhoA is also expressed in intramuscular stem cells including satellite cells and plays a role in fate specification of these cells is not clear so far. Recent studies showed that inhibition of the Rho signaling pathway by a chemical inhibitor or a microgravity condition induces adipogenic differentiation of human mesenchymal stem cells (McBeath et al., 2004; Meyers et al., 2005). In addition, mouse embryo-derived fibroblasts (MEFs) lacking p190-B RhoGAP, a Rho inhibitory protein, do not undergo adipogenic differentiation. On the other hand, deletion of p190-B RhoGAP in MEFs induces differentiation into myogenic cells in response to IGF-I (Sordella et al., 2003). These observations suggest that the Rho signaling pathway is involved in myogenesis-adipogenesis determination of mesenchymal cells.

Although the mechanisms of fatty infiltration in skeletal muscle and the cellular origin of accumulated adipocytes in some pathological conditions are unclear, the fact that degenerative or atrophic fibers are persistently present in fat accumulated-muscle indicates the possibility that muscle fiber condition would be one of the niches affecting lineage specification of intramuscular cells. So, we hypothesize that there is the interaction between muscle fiber condition and fat accumulation in muscle, and this interaction is mediated by RhoA signaling pathway.

2. Materials and methods

2.1. Animals

Male Wistar-Imamichi rats (2–4 months old) were purchased from the Institute for Animal Reproduction (Ibaraki, Japan). Male C57BL/6J mice (8-weeks old) were purchased from Charles River Laboratories (Yokohama, Japan). They were housed in a temperature-controlled room with a 14 h light and 10 h dark cycle (light on at 5:00 am). Food and water were provided *ad libitum*. All animal experiments in this study were performed according to the Guideline for the Care and Use of Laboratory Animals, The University of Tokyo.

2.2. Single fiber culture

Single fiber culture was carried out according to Bischoff (1986), and Shefer and Yablonka-Reuveni (2005). Briefly, single muscle fibers with attached satellite cells were isolated from the hindlimb *extensor digitrum longus* (EDL) muscle of adult male rats. The EDL muscle was carefully removed at the tendon and treated with 0.2% (w/v) collagenase type I (Sigma, MO) reconstituted in Dulbecco's Eagle Medium (DMEM) (Invitrogen, CA) at 37 °C for 90 min with slight shaking. After several gentle triturations with a wide-bore pipette, the muscle was further treated with enzyme at 37 °C for 10 min. The muscle was transferred into fresh 10% FBS/DMEM, and fine fibers were liberated from the muscle by gentle trituration with a wide-bore pipette. Then, fine fibers were treated with vigorous pipetting to induce contraction of fibers. Fibers were sequentially washed three times in DMEM to remove interstitial and endothelial cells. Hereafter, fine fibers and contracted fibers are referred to as intact fibers (IF) and hyper-contracted fibers (HCF), respectively. Both fibers were maintained as non-adherent cultures in 10% FBS/DMEM on 24-well non-coated culture plates (IWAKI, Tokyo, Japan) for 5–7 days, and then stained for specific markers.

2.3. Primary antibodies

The following primary antibodies were used: Anti-Pax7, mouse monoclonal (1:250 dilution; Developmental Studies Hybridoma Bank (DSHB), IA); anti-MyoD1, mouse monoclonal (1:100; 5.8A, Novocastra, Newcastle upon Tyne, UK); anti-PPARγ2 mouse monoclonal (1:200; Santa Cruz, CA); anti-laminin, rabbit polyclonal (1:1000; Sigma); anti-myosin heavy chain (1:20; MF20, DSHB), anti-RhoA, mouse monoclonal (1:500; 26C4, Santa Cruz); anti-M-cadherin antibody rabbit polyclonal (1:10 000; kindly gifted from Dr. Shin'ichi Takeda, National Center of Neurology and Psychiatry, Tokyo, Japan).

2.4. Immunocytochemistry

Cells on fibers and culture dishes were fixed with 4% paraformaldehyde (PFA) in phosphate buffed saline (PBS) for 15 min at room temperature (RT). Cells were washed with PBS and incubated in 5% normal goat serum (NGS; Zymed, CA) and 0.1% Triton-X (Sigma) in PBS to block non-specific binding of antibodies. After cells were washed in PBS, primary antibodies were applied, and cells were incubated overnight at 4 °C. After washing cells with PBS, Alexafluor 594 or 488 conjugated anti-mouse IgG (1:400 diluted with 5% NGS in PBS; Invitrogen) was added and incubated for 1 h at RT. Cell nuclei were counterstained with Hoechst 33258. In some experiments, mature adipogenic cells were visualized by Oil-Red O (OR) staining after immunostaining. Briefly, cells fixed with 4% PFA were stained in OR solution (Sigma, 0.5% (w/v)) for 7 min at RT, then washed in PBS.

Quantitative analyses of cells detached from single muscle fiber were performed. Total number of cells and number of cells on the dish positive for anti-PPARγ antibody, MyoD antibody and OR, obtained from seven randomly chosen different microscopic fields using a 20× objective, were counted. Cell number and percentage of positive cells were averaged for the triplicate culture wells. Alternatively, number of positive cells for anti-MyoD and -PPARγ antibodies, and number of OR-positive cells on fiber obtained from 10 fibers chosen randomly per well, were counted for the triplicate wells.

2.5. Thrombin and Y-27632 administration

Thrombin (Sigma) was dissolved in DMEM and added into 10% FBS/DMEM medium (1, 5 and 10 units/500 μl of medium). HCF were cultured in thrombin-containing medium during the first 3 days and then cultured in 10% FBS/DMEM for 4 additional days. Number of MyoD-positive myogenic cells and OR-positive adipogenic cells on fibers and dish were counted at the end of culture (day 7). Fusion index was estimated from number of nuclei in MHC-positive cells per all nuclei in the field at day 10.

Y-27632 solution (10 μM, Calbiochem, CA), a specific inhibitor of ROCK (Ishizaki et al., 2000), was added to 10% FBS/DMEM during the first 3 days to inhibit intracellular RhoA signaling, and the fiber was removed from the well on day 4. Cells detached from