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Rapid Communication

Prospective heterotopic ossification progenitors in adult human skeletal muscle

Jennifer Downey [a], Dominique Lauzier [b], Peter Kloen [c], Klaus Klarskov [a,d], Martin Richter [a,e], Reggie Hamdy [b,f], Nathalie Faucheux [a,g], Anthony Scimè [h], Frédéric Balg [a,i], Guillaume Grenier [a,i,*]

[a] CHUS Clinical Research Centre, Université de Sherbrooke, Sherbrooke, QC, Canada
[b] Shriners Hospital for Children, Montreal, QC, Canada
[c] Department of Orthopedic Surgery, Academic Medical Centre, Amsterdam, The Netherlands
[d] Department of Pharmacology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, QC, Canada
[e] Department of Pediatrics, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, QC, Canada
[f] Department of Surgery, Orthopedic Surgery Division, McGill University, Montreal, QC, Canada
[g] Department of Chemical and Biotechnological Engineering, Faculty of Engineering, Université de Sherbrooke, Sherbrooke, QC, Canada
[h] Stem Cell Research Group, Faculty of Health, York University, Toronto, ON, Canada
[i] Department of Orthopedic Surgery, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, QC, Canada

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ABSTRACT

Skeletal muscle has strong regenerative capabilities. However, failed regeneration can lead to complications where aberrant tissue forms as is the case with heterotopic ossification (HO), in which chondrocytes, osteoblasts and white and brown adipocytes can arise following severe trauma. In humans, the various HO cell types likely originate from multipotent mesenchymal stromal cells (MSCs) in skeletal muscle, which have not been identified in humans until now. In the present study, adherent cells from freshly digested skeletal muscle tissue were expanded in defined culture medium and were FACS-enriched for the $CD73^{+}CD105^{+}CD90^{-}$ population, which displayed robust multilineage potential. Clonal differentiation assays confirmed that all three lineages originated from a single multipotent progenitor. In addition to differentiating into typical HO lineages, human muscle resident MSCs (hmrMSCs) also differentiated into brown adipocytes expressing uncoupling protein 1 (UCP1). Characterizing this novel multipotent hmrMSC population with a brown adipocyte differentiation capacity has enhanced our understanding of the contribution of non-myogenic progenitor cells to regeneration and aberrant tissue formation in human skeletal muscle.

Introduction

Skeletal muscle possesses a remarkable capacity to regenerate following trauma, mainly through myogenic stem cells [1]. However, efficient tissue repair also requires the activation of resident cells within the stroma, notably mesenchymal stromal cells (MSCs). Inappropriate activation can lead to aberrant tissue formation such as heterotopic ossification (HO), where extra-skeletal bone forms, most commonly in muscle, through an endochondral process [2–4]. While HO can arise from *fibrodysplasia ossificans progressiva* (FOP), an uncommon hereditary disease, most cases result from a local trauma (surgery, muscular trauma, fractures) or neurological injury [5]. Traumatic HO has been thought to result from the inappropriate differentiation of muscle-resident progenitor cells, induced by a pathological imbalance of local or systemic factors [6]. However, the cellular origin of the ectopic bone in HO remains a matter of debate [7].

While local muscle resident MSCs are a logical candidate as HO progenitors, other cells have been proposed. Some studies have implicated vascular endothelial cells as a potential source for HO progenitors [8]. Constitutively activated ACVRI in FOP change the morphology of endothelial cells to mesenchymal-like cells and induce the co-expression of mesenchymal markers *in vitro*, a process that resembles the endothelial–mesenchymal transition [8]. Moreover, endothelial marker Tie2 has been histologically observed in heterotopic lesions from patients with FOP. In addition, lineage tracing studies using Tie2-Cre reporter mice indicated that these cells generate approximately half the chondrocytes and osteoblasts found in skeletal muscle lesions [8,9]. However, Tie2 is not specific to endothelial cells and is also expressed in a number of non-endothelial cell types, including perivascular cells [10,11]. It has also been shown *in vivo* that the endothelial fraction of murine Tie2

Abbreviations: HO, heterotopic ossification; MSC, mesenchymal stromal cell; hmrMSC, human muscle resident MSC; UCP1, uncoupling protein 1.

* Corresponding author at: 3001–12th Avenue North, Sherbrooke, QC, Canada, J1H 5N4. Fax: +1 819 820 6410.
E-mail address: guillaume.grenier@usherbrooke.ca (G. Grenier).

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cells ($Tie2^+CD31^+$) does not participate in HO whereas the non-endothelial fraction of Tie2 cells ($Tie2^+CD31^-$) does [12]. These recently published findings strongly suggest that the Tie2 progenitors observed in HO are not of endothelial origin [7]. Indeed, more than 90% of $Tie2^+CD31^-$ cells are also $PDGFR\alpha^+Sca1^+$, pointing to a mesenchymal rather than an endothelial origin [12], which supports the findings of Leblanc et al., who showed that a $Sca1^+CD31^-$ muscle resident stromal cell population contributes to HO [2].

In humans, PDGFRα has been reported to be a specific marker for interstitial mesenchymal progenitors that are distinct from $CD56^+$ myogenic cells and that possess adipogenic and fibrogenic potentials [13]. While human skeletal muscle $PDGFR\alpha^+$ cells display osteogenic potential *in vivo* [14], the confirmation of their osteogenic activity came from subcutaneous-implanted cell-loaded PLGA-hydroxyapatite blocks, which are not likely representative of the HO environment. In addition, their osteogenic activity was comparable to CD56 myogenic cells [14], suggesting that PDGFRα may not be a marker that is exclusive to osteogenic progenitors. Other human studies have shown that a fraction of skeletal muscle adherent cells can give rise to osteoblasts and that this potential is greatly increased following trauma [15,16]. A multipotent myo-endothelial cell population in human skeletal muscle has been characterized based on the presence of myogenic (CD56) and endothelial (CD34, CD144) cell surface markers and the ability to differentiate into mesenchymal lineages [17].

Interestingly, the brown adipogenic potential of these putative HO progenitors has not been investigated, although it has been shown that brown adipocytes can promote endochondral ossification in an HO mouse model by regulating oxygen availability and inducing a hypoxic microenvironment [18,19]. Until now, an adult human skeletal muscle-derived progenitor cell population giving rise to brown adipocytes has not been characterized. While uncoupling protein 1 (UCP1) mRNA expression in adult human whole skeletal muscle has been reported, the identity of the responsible progenitors is not known [20].

Given the varied tissue make-up of HO, no adult human skeletal muscle resident progenitor cells have been identified that can differentiate into mesenchymal as well as brown adipogenic lineages. We enriched human muscle resident mesenchymal stromal cells (hmrMSCs) and, for the first time, showed that hmrMSCs are clonally capable of efficient differentiation toward osteogenic, chondrogenic and adipogenic lineages. Interestingly, these hmrMSCs were also able to differentiate into UCP1-expressing brown adipocytes, cells that we also detected in human HO samples, which lends credence to a possible role for them in the development of HO. A better understanding of the cellular origin responsible for HO will provide a potential therapeutic target to treat, mitigate, or prevent this debilitating condition.

Materials and methods

Human tissue

Healthy human skeletal muscle tissue samples (gracilis and semitendinosus) were obtained from patients (34 ± 8 years of age; 54% male and 46% female) undergoing anterior cruciate ligament reconstruction surgery. HO tissue was obtained from a 21-year-old male patient who had developed a mass in the gluteal muscle following a mid-shaft femur fracture (Table S1). The samples were collected following resection surgery. The protocols were approved by the Centre Hospitalier de l'Université de Sherbrooke Ethics Committee (#11-122 and #13-164), and written consent was obtained from the patients.

Cell isolation and culture

Carefully dissected skeletal muscle samples were minced and then digested for 30 min at 37 °C with 1 mg/mL of collagenase type I (Sigma) in DMEM containing 10% FBS. The tissue slurry was diluted with medium, passed through 70-μm and 40-μm cell strainers (Becton Dickenson) and centrifuged at 325 g for 6 min at 4 °C. Primary human skeletal muscle cells were seeded in tissue culture plates coated with Mesencult-SF® attachment substrate and were expanded as adherent cells in Mesencult-XF® medium (StemCell Technologies). After 7 days, an average of 7×10^5 adherent cells were recovered per gram of tissue. The cells were trypsinized at 80% confluence and were centrifuged and resuspended in Mesencult-XF® medium as first passage cells, with fresh medium changes every 3–4 days. The cells were sub-cultured at a density of 4×10^3 cells/cm^2.

Fluorescent activated cell sorting

First passage cells were detached with the Accutase™ Cell Detachment solution (BD Biosciences), centrifuged and resuspended at ~1×10^6 cells per ml in cold sorting buffer (PBS, 1 mM EDTA, 25 mM HEPES, pH 7.0, 1% FBS). The cells were incubated for 20 min on ice with the appropriate primary antibodies (Table S2) according to the manufacturers' instructions. During the cell sorting experiment, live cells were distinguished from dead cells using LIVE/DEAD® Violet Viability/Vitality kits (Invitrogen). Fluorescence was compensated using BD CompBeads Set Anti-Mouse Ig, κ (BD Biosciences). The cells were sorted a BD FACSAria™ cell sorter (BD Biosciences) equipped with four lasers and a 100-μm nozzle set at 20 psi. Sorting gates were defined based on unstained controls. The cells were analyzed using FlowJo 7.9 software (Treestar Inc.). A population of unsorted cells was used as a control. Unsorted and sorted fractions were then expanded as described above.

Differentiation protocols

The osteogenic, chondrogenic and white and brown adipogenic differentiation protocols were adapted from published protocols [21-24] and are presented in Table S3. Briefly, for the osteogenic, adipogenic (white and brown) and myofibroblastic assays, the cells were seeded at a density of 8×10^3 cells per well in 24-well collagen-coated (Millipore) plates (4000 cells/cm^2) in Mesencult-XF® medium and incubated at 37 °C in a CO_2 incubator until they reached confluence.

For osteogenic differentiation, the cells were cultured in osteogenic medium (Table S3) for 21 days. Unstimulated cells were cultured in osteogenic basal medium (DMEM, 5% horse serum [HS]). To assess mineralization, calcium deposits in cultures were stained with 40 mM Alizarin Red-S, pH 4.1).

For white adipogenic differentiation, the cells were cultured in adipogenic induction medium for 3 days and then in adipogenic growth medium (Table S3) for a further 18 days for oil Red O staining, or 11 days for gene expression analyses. Unstimulated cells were cultured in adipogenic induction/growth basal medium (DMEM, 3%/10% FBS). An oil red O solution (0.5% oil red O in isopropyl alcohol; Sigma) was used to detect triglycerides in the lipid droplets of mature adipocytes.

Alizarin red- and oil red O-stained area was quantified using ImageJ software (version 1.46, National Institute of Health) [25].

For brown adipogenic differentiation, the cells were incubated in adipogenic induction medium for 3 days and then in brown adipogenic growth medium (Table S3) for a further 11 days. Unstimulated cells were cultured in the same adipogenic basal media as the stimulated cells (DMEM, 3%/10% FBS).

To stimulate chondrogenesis, ~2.5×10^5 cells were pelleted by centrifugation (350 g, 6 min, 4 °C) and were resuspended in chondrogenic culture medium (Table S3). Unstimulated cells were cultured in chondrogenic basal medium (serum-free DMEM). The cells were harvested by centrifugation on day 21. The pellets were fixed in 4% phosphate buffered formalin and were embedded in paraffin. Sections (5 μm) cut using an HM325 microtome (Micron) were immersed in an Alcian blue solution (1% Alcian blue in 3% acetic acid; Acros Organics) to stain highly sulfated proteoglycans that characterize the cartilaginous matrix.