Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle

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Putative myogenic and endothelial (myo-endothelial) cell progenitors were identified in the interstitial spaces of murine skeletal muscle by immunohistochemistry and immuno-electron microscopy using CD34 antigen. Enzymatically isolated cells were characterized by fluorescence-activated cell sorting on the basis of cell surface antigen expression, and were sorted as a CD34+ and CD45− fraction. Cells in this fraction were ~94% positive for Sca-1, and mostly negative (<3% positive) for CD14, 31, 49, 144, c-kit, and FLK-1. The CD34+/45− cells formed colonies in clonal cell cultures and colony-forming units displayed the potential to differentiate into adipocytes, endothelial, and myogenic cells. The CD34+/45− cells fully differentiated into vascular endothelial cells and skeletal muscle fibers in vivo after transplantation. Immediately after sorting, CD34+/45− cells expressed only c-met mRNA, and did not express any other myogenic cell-related markers such as MyoD, myf-5, myf-6, myogenin, M-cadherin, Pax-3, and Pax-7. However, after 3 d of culture, these cells expressed mRNA for all myogenic markers. CD34+/45− cells were distinct from satellite cells, as they expressed Bcrp1/ABCG2 gene mRNA (Zhou et al., 2001). These findings suggest that myo-endothelial progenitors reside in the interstitial spaces of mammalian skeletal muscles, and that they can potentially contribute to postnatal skeletal muscle growth.

Introduction

Recent identification of tissue specific stem cells in adult brain (Reynolds and Weiss, 1992; Johansson et al., 1999), bone marrow (Prockop, 1997; Ferrari et al., 1998), and skeletal muscle (Gussoni et al., 1999; Jackson et al., 1999) opens new pathways for tissue reconstitution therapy via cell transplantation. For skeletal muscle, the satellite cells, residing between the basal lamina and the plasma membrane of muscle fibers, have been considered the only myogenic source for postnatal growth, repair, and maintenance of skeletal muscle (Grounds, 1991; Schultz, 1996). Recently, a hematopoietic and myogenic stem cell population in the muscle called side population (SP) cells (Beauchamp et al., 2000; Lee et al., 2000; Torrente et al., 2001), and the muscle SP cells have also been reported to be a mixture of CD34-positive and

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-negative (Gussoni et al., 1999) cells. Therefore, it is possible that more primitive cells, distinct from satellite cells, reside in the interstitial spaces, and that these cells differentiate into myogenic cells and contribute to new fiber formation. To further investigate the role of interstitial CD34+ cells in the muscle, we switched our experimental

**Figure 1. Localization of CD34+ cells in the interstitial spaces of skeletal muscles of 3-wk-old mice.** Small mononucleated CD34+ cells were observed in the interstitial spaces (surrounding brown reaction, arrows in A–D). The CD34+ cells are located outside of the basal lamina (laminin staining, dark purple). The location, outside of the basal lamina was also confirmed by immunoelectron micrograph (E and inset). The CD34+ cell shows lower nuclear/cytoplasmic ratio representing immature cell. Reaction products of DAB can be seen in surface of CD34+ cell as well as in the lumina of endothelial cell (arrowheads in E). However, note that the satellite cells shows CD34–, whereas CD34+ cell can be seen within the interstitial spaces (F). Inset in F shows higher magnifications of the corresponding square areas on the photograph. Cell membrane of both satellite cell and muscle cell is evident (arrowheads). Immunofluorescent costaining with CD34 (G, Rhodamine-red) and CD45 (J, FITC-green) shows that CD34+ cells are CD45+ (G and J, arrows), and they are located in the interstitial spaces (H and I, arrows). Vasculature related CD34+ reactions are also observed in enclosed portions in G and H. Staining in the marrow of the tibia on the same sections with A–C and with G–J are shown in D (CD34, brown) and K (CD34, red and CD45, green) as a positive control. The mean frequencies of CD34+ and CD45+ mononuclear cells appearing in a unit area of 3-wk-old mice muscles were 1.9 (±0.3, SE) and 2.9 (±0.4), respectively. The data were obtained from four to six unit areas (175 x 130 μm per unit area) in 20 sections taken from three mice, i.e., corresponding to ~40% of the total interstitial cells in the unit area. Note that there were no CD34+ cells inside the basal lamina. Green reactions in A–D, nuclear stain with methyl green. Blue staining in G and J, nuclear stain with DAPI. HE, hematoxylin-eosin staining; TV, transmission view of confocal laser scanning microscope; 34+, CD34 positive cell; EC, endothelial cell; Cap, capillary; SC, satellite cell; MN, myonuclear. Bars, (A–D and G–J) 10 μm; (E and F) 1 μm; (K) 25 μm.

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572 The Journal of Cell Biology | Volume 157, Number 4, 2002
model from rats to mice because of the large number of cell surface markers available for mice.

**Results and discussion**

Immunohistochemical staining with anti-CD34 and laminin (a marker for the basal lamina) on the entire lower hind limb of 3-wk-old mice revealed numerous CD34+ cells outside the basal lamina, i.e., a location distinct from that of satellite cells (Fig. 1, A–D). Positive staining in the marrow cells of the tibia (Fig. 1 D, used as a positive control) was evident; however, there were no CD34-positive reactions within the basal lamina where satellite cells would be located normally. A location of CD34+ cell that outside the basal lamina was also confirmed by immunoelectron microscopy, representing immature shape with a less cytoplasm and cell-membrane.

To examine the CD34+ cells in the interstitial spaces of skeletal muscles, we obtained the interstitial cell enzymatically extracted cells (EECs) by their reaction to CD34 and CD45 antigens (Fig. 2). The EEC was divided into four subpopulations, CD34+/H11001, CD34+/H11001, CD34+/H11002, and CD34+/H11002 (Fig. 2 C). The CD45-negative fractions (CD34+/H11001 and CD34+/H11002) were considered to be hematopoietic cells mainly consisting of monocytes and/or macrophages (Thomas, 1989), and were not used in this study. However, the possibility still remains that CD34-positive cells are endothelial cells (Asahara et al., 1997). Because the skeletal muscle-derived CD34+/H11001 fraction (Sk-34) may include endothelial cells, we characterized them further by endothelial cell related markers such as CD14, 31, 49, and 144, c-kit, FLK-1, and mostly negative for all other markers (≥3% positive cells). To determine the growth characteristics and multilineage differentiation of purified Sk-34 cells in vitro, we performed clonal cell culture in a semisolid medium. The Sk-34 cells underwent cell division and/or myoblast fusion and used only the extracted fractions, i.e., all remaining cell surface markers. We used an extremely mild enzymatic digestion based on the living single fiber isolation method characterized them further by endothelial cell related markers such as CD14, 31, 49, and 144, c-kit, FLK-1, and mostly negative for all other markers (≥3% positive cells) for CD31, FLK-1, and CD44 (VE-cadherin) (Asahara et al., 1997, 1999; Yamashita et al., 2000). Therefore, the Sk-34 fraction contained very few hematopoietic and endothelial cells. The muscle SP cells, a hematopoietic and myogenic stem cell population in skeletal muscle (Gussoni et al., 1999; Jackson et al., 1999), are also positive for Sca-1 and negative for c-Kit and CD45. However, the muscle SP cells are a mixture of CD34-positive and -negative (Gussoni et al., 1999) cells, thus they might be sorted both in the CD34+/H11001 and CD34+/H11002 fraction.

To characterize the cells comprising these colonies after 6 d of culture, floating and/or weakly attaching cells in individual colonies were picked up by micropipette and made cytospins and stained for MyoD. The majority of the colonies were positive for MyoD (Fig. 2 F) and mostly negative for all other markers (≥3% positive cells) (Fig. 2, D–E and G–J). Endothelial progenitor cells are positive for Sca-1 as are the Sk-34 cells, but also are positive for CD31, FLK-1, and CD44 (VE-cadherin) (Asahara et al., 1997, 1999; Yamashita et al., 2000). Therefore, the Sk-34 fraction contained very few hematopoietic and endothelial cells. The muscle SP cells, a hematopoietic and myogenic stem cell population in skeletal muscle (Gussoni et al., 1999; Jackson et al., 1999), also are positive for Sca-1 and negative for c-Kit and CD45. However, the muscle SP cells are a mixture of CD34-positive and -negative (Gussoni et al., 1999) cells, thus they might be sorted both in the CD34+/H11001 and CD34+/H11002 fraction.

To determine the growth characteristics and multilineage differentiation of purified Sk-34 cells in vitro, we performed clonal cell culture in a semisolid medium. The Sk-34 cells began to form colonies within 4–5 d when cultured in medium containing methylcellulose (Fig. 3 A). As the number of cells gradually increased, two populations of cells were found in a colony at day 6: a floating and/or weakly attaching cell population and an adhesive and spreading cell population (Fig. 3 B, arrows). Both cell populations continuously increased their numbers (Fig. 3 C), and small tubes were observed after 10 d of culture (Fig. 3 C, arrowheads). At this time, the colonies consisted of large, round cells and small tubes showing spontaneous and intermittent contractions (Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200112106). After 14 d of culture, many myotubes were formed in the colony (Fig. 3 D) and these also contracted actively and spontaneously (Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200112106).

To characterize the cells comprising these colonies after 6 d of culture, floating and/or weakly attaching cells in individual colonies were picked up by micropipette and made cytospins and stained for MyoD. The majority of the cells were positive for MyoD (Fig. 2 G). For the remaining adhesive and spreading cell populations, the medium containing methylcellulose was washed out by DME containing 5% FCS, and the cells prepared for analyzing the uptake of DiL-
Ac-LDL (a functional marker for endothelial cells and macrophages). Several cells demonstrated uptake of Dil-Ac-LDL, indicating a differentiation to endothelial cells (Fig. 3, E and F, arrows). The same procedures were performed for the samples after 10 d of culture, and the remaining adhesive and spreading cells were stained with oil-red O, a marker of lipid deposition. Several cells showed oil droplet-like staining typical of fat cells (Fig. 3 H, arrows). Combined, these observations confirmed the multilineage potential (myogenic, endothelial, and adipogenic) of a colony-forming unit (CFU) in the Sk-34 cells in vitro. The colonies containing three lineage was formed 75 ± 58 out of 108 ± 61 total colonies when 1 × 10^4 cells were plated. Remaining 30 colonies were composed by endothelial and adipogenic cell populations, and there were no colonies composed by exclusively single population such as myogenic, endothelial, or adipogenic cell. Interestingly, our preliminary data using Hoechst 33342 suggested that Sk-34 cells included about 0.6% of SP cells (unpublished data) as similar to a percentage of the CFU in Sk-34 cells (0.75 to 1.1%). To further examine the potential of Sk-34 cells, we performed clonal cell culture on soft agar–coated dishes to avoid cell attachment to the plates. After 7 d of culture, a large number of floating colonies appeared (Fig. 3, I and J). Several cells in these colonies fused to each other and formed sphere-like shapes (Fig. 3 I), and others remained as individual in a colony (Fig. 3 J). Interestingly, the double negative fraction (CD34^-/45^- cells) did not grow well in the same clonal methylcellulose culture in contrast to the Sk-34 cells, and the rate of myotube formation in the Sk-34 cells apparently was reduced in a liquid culture system (unpublished data).

To evaluate the ability of Sk-34 cells to reconstitute muscles in vivo, we prepared Sk-34 and CD34^-/45^- cells from GFP transgenic mice (Okabe et al., 1997) and transplanted them into the tibialis anterior (TA) muscles of male immune-deficient NOD/scid mice. 6 wk after the injection, engrafted anti–GFP-positive cells were observed in the TA muscles of three of the five Sk-34–injected mice, but in none of the CD34^-/45^- injected mice. Injected donor cells differentiated into complete muscle fibers (Fig. 3, K and L, arrowhead) and vascular endothelial cells (Fig. 3, K and L, arrows) in the recipient muscles. The combined results of the culture and transplantation experiments indicate that purified Sk-34 cells can differentiate into myogenic, endothelial and fat cells in vitro and fully differentiate into skeletal muscle and vascular endothelial cells in vivo.

To assess the myogenic profile of the Sk-34 cells, we examined the expression of myogenic cell related gene mRNAs. The
CD34+ cells clearly located outside of the basal lamina of the muscle fibers, whereas no CD34+ cells were detected in the satellite cell position beneath the basal lamina (Fig. 1, A–F). The reasons for this discrepancy are unknown. However, it is likely that the CD34+ cells identified in the present study are a different population of cells than those reported elsewhere, as the expression patterns for specific markers are different. For example, Beauchamp et al. (2000) reported that ~80% of the satellite cells associated with isolated single muscle fibers were CD34+, myf-5+, and M-cadherin-positive, and a yet undefined minority population of satellite cells were negative for all three markers. These characteristics do not correspond to the properties of the Sk-34 cells described in the present paper.

We also found that Sk-34 cells are Pax3 and Pax7 negative on day 0, but positive after 3 d of culture. Seale et al. (2000), demonstrating that there was an absence of satellite cells in the skeletal muscles of Pax7 knockout mice, whereas muscle SP cells were present. Their finding suggested that the induction of Pax7 in muscle-derived stem cells induced satellite cell specification by restricting alternate developmental programs and clearly demonstrated that satellite cells were a distinct population from muscle SP cells. Furthermore, these data suggested that muscle SP cells may form a reservoir of satellite cells during the latter stages of embryonic muscle development and may persist in adult skeletal muscle to maintain a steady-state number of satellite cells (Seale et al., 2000). The recent work of Zhou et al. (2001) demonstrated that Bcrp1/ABCG2 gene is an important determinant of the SP phenotype, and the non-SP fraction (including satellite cells) did not express Bcrp1 mRNA in mouse muscle. We also examined the expression of Bcrp1 mRNA in the Sk-34 (d-0 and CFU of d-4) and CD34+/45− (d-0) fraction using RT-PCR analysis (Fig. 4 B). Both the Sk-34 (d-0) and CD34+/45− (d-0) fractions expressed Bcrp1 mRNA. In addition, the CFU of Sk-34 cells expressed both Bcrp1 and MyoD, indicating that Bcrp1-positive cells differentiated into myogenic cells. In this regard, it is likely that the Sk-34 cell populations included one of the phenotypes of muscle SP cell populations, and that CD34+/45− fractions also included SP cell fractions. Combined, these results suggest that Sk-34 cells are distinct from satellite cells, as they express Bcrp1 mRNA. However, it is likely that Sk-34 cells have the potential to become satellite cells in newly formed fibers since they have the ability to express Pax7 after 3 d of culture.

Recent work suggests that satellite cells can be derived from endothelial precursors associated with the embryonic vasculature (De Angelis et al., 1999). Seale et al. (2000) also suggest that progenitor cells associated with the embryonic vasculature, either directly or indirectly, give rise to satellite cells during the embryonic stage of development. These authors further suggest that putative vasculature-associated precursors could give rise to pluripotent stem cells in the adult. Young et al. (1995, 1999) also proposed that there were mesenchymal stem cells within the connective tissues of fetal and adult human skeletal muscle and several tissues and organs on the chick embryo. There are consistent with the possibility that embryonic myo-endothelial progenitor cells could remain in the interstitial spaces of skeletal muscles and express CD34 as observed in the Sk-34 cells.
Based on these observations, we conclude that Sk-34 cells are putative myo-endothelial progenitor cells located in the interstitial spaces of skeletal muscle and are distinct from satellite cells. It is possible that Sk-34 cells are a reservoir of satellite cells and one of the phenotypes of muscle SP cell populations remaining in the interstitial spaces of postnatal and/or adult skeletal muscle. After all, in the present study, we first demonstrated in vivo location of newly myogenic cell populations distinct from satellite cells, and possibly they are one of phenotypes of muscle SP cells. Our finding of myo-endothelial progenitors in skeletal muscle may provide important new insight into muscle cell biology, and possible therapeutic strategies for cell transplantation studies. Tissue specific stem cells, including Sk-34 cells, may share a common embryonic origin and possess the capacity to activate diverse genetic programs in response to peculiar environmental stimulation.

Materials and methods

Mouse strain

C57BL/6 mice were used for the cell cultures, immunohistochemistry, immunoelectron microscopy, and flow cytometric characterizations. Green fluorescent protein transgenic mice (C57BL/6 Tg(Ncat EGFPlbY01)) were provided by Dr. M. Okabe (Osaka University, Osaka, Japan) and used in cell transplantation studies as donor mice, and NOD/Shi-scid mice as recipients.

Cell purification and characterization

Intercellular cells were harvested from the thigh and lower leg muscles of 3-wk-old mice based on an isolation method for intact, living single muscle fibers associated with satellite cells described previously (Bischoff, 1986). Muscles were treated with 0.06% collagenase type IA (Sigma-Aldrich) in DME containing 10% FCS with gentle agitation for 2 h at 37°C. Extracted cells were filtered through a 40 and then a 20 μm nylon mesh, washed, and resuspended in DME containing 20% FCS. The cells were stained with a combination of FITC-, phycoerythrin-, and biotin-conjugated anti-mouse CD34 (RAM34), CD45 (30-F11), CD117 (c-kit, 2B8), FLK-1 (Ly-73), Sca-1 (Ly6A/E), and CD31 (MEC13.3), and resuspended in DME containing 20% FCS. CD34 cells were purified from hindlimb muscles of GFP transgenic mice, and 2 × 10⁴ cells were injected directly into the TA muscle of NOD/scid mice (n = 5 for each cell). For detection of engrafted Sk-34 cells from GFP transgenic mice, recipient mice were perfused with warm ringer and 4% PFA/PB. The TA muscles were removed and immersed in 4% PFA/PB overnight. Samples were treated by graded suroce (5-25%) PBS and then quick frozen in isopentane. Serial 7-μm thick cross-sections (20-25 sections) were cut. Immunostaining was performed using rat anti-GFP monoclonal antibody (JFP-K2), produced by S. C. Fujita and colleagues at the Mitsubishi Institute of Life Sciences (Tokyo, Japan), 1:10 for 2 h at room temperature. Reactions were visualized by streptavidin–biotin complex and DAB.

RT-PCR

Total RNA was extracted from EEC, Sk-34 and CD34+/45+ fractions using a total RNA isolation kit (Wako Pure Chemical). Equal amounts of RNA were reverse-transcribed using the RNA-PCR kit version 2.1 (Takara). The primed paired results were as follows: mouse MyoD: AAG CAT ACT TGA CAC GCC CCG A/AGA CCT TCG ATG CGG ATG G (451 base pair); myf-5: GTC AAC CAA GGT TTC GAG ACG/CGG AGC TTT TAT CTG CAC GAC (305 base pair); myf-6: ATT CTG CGG AGT GCC ATC ATG TGT TCC AAA TCG TGG ATG (356 base pair); Myogenin: TAC GTC CAT CTT GGA CAT CAG CAC GGT GAA TAT Acc GTG CCT CTC TCT GGT (359 base pair); c-met: CCA ACC GCG GTA TGT CAG CAG GCC CCG A/AGA CCT TCG ATG TAG CGG ATG G (451 base pair); Pax-3: CCT GGA ACC CAC GAC GGT GAC GTG GCT GAA CAC CCA GCC ATT TAG TAC GTA/AAAG GAC GGG TGG AAG AAA AGC GCC (409 base pair) was used as the control. The samples were denatured at 94°C for 5 min, followed by amplification rounds consisting of 94°C for 30 s denaturing, 65°C for 30 s annealing, and 72°C for 30 s (extension) for 30 cycles, and 72°C for 10 min. For Fig. 4 A, cells were cultured in DME containing 20% FCS without methylcellulose for 3 d, and for Fig. 4 B, Sk-34 cells were cultured in methylcellulose medium for 4 d.

Online supplemental material

Videos 1 and 2, available online at http://www.jcb.org/cgi/content/full/jcb.200112106; show spontaneous, intermittent, and active contractions of large, round cells in Fig. 3 C and many myotubes in Fig. 3 D.

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