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ORIGINAL ARTICLE

Quantitative and qualitative *in vitro* analysis of the stem cell potential of hematopoietic cells purified from murine skeletal muscle

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The murine skeletal muscle contains hematopoietic stem cells, but this potential has so far not been studied quantitatively or qualitatively *in vitro*. To quantify the hematopoietic stem cell potential, we have used highly purified SP/CD45^{+} cells in long-term culture initiating cell (LTC-IC) assays. The SP/CD45^{+} cell population purified from murine muscle was found to have significant stem cell activity with an LTC-IC frequency of 1/640. Single-cell-sorted SP/CD45^{+} cells from muscle exhibited robust proliferative activity *in vitro* at day 16 (380-fold amplification), especially after culture with OP-9 layers that also support embryonic stem cells. Amplified cell populations originating from single cells exhibited multilineage differentiation ability with evidence of myeloid, lymphoid and NK cell markers. Thus, our results demonstrate that hematopoietic stem cells that can be quantified by LTC-IC assays exist in the murine skeletal muscle and show also for the first time, at the single-cell level, that these cells exhibit multilineage differentiation ability and major proliferative potential.

Keywords: hematopoiesis, stem cells, muscle

Introduction

Recent data from several laboratories have demonstrated the presence of hematopoietic potential in murine skeletal muscle [1-3]. This potential, which has been shown to be essentially due to cells with a CD45^{+} phenotype [4-6], was enhanced by the use of either Sca-1 or SP (side population) markers [1, 7]. These cells have been shown to give rise to long-term hematopoiesis [2], and their ability to generate hematopoietic cells in at least three generations of lethally irradiated recipients for more than 1 year suggests a self-renewal ability [3]. The contamination of muscle tissue by a circulating stem cell has been suggested as a possible explanation for this phenomenon [5], but the transplantation of many nucleated blood cells did not yield any survival in irradiated mice [5, 8, 9]. The current hypothesis is that there is a hematopoietic cell niche in the muscle tissue that is either of post-natal or embryonic origin. To further characterize the hematopoietic potential of these cells, we have tested CD45^{+} cells and their SP/CD45^{+} subsets in *in vitro* assays. Our results document for the first time the long-term culture initiating cell (LTC-IC) frequencies in murine muscle and the detection of extensive proliferative and multilineage differentiative ability in muscle-derived SP/CD45^{+} cell populations at the single-cell level.

Materials and Methods

Mice

All experiments were performed at the animal facility of the

Gustave Roussy Institute, under the appropriate housing and feeding conditions for the animals. In all experiments, C57Bl/6 mice (Harlan laboratories, Gannat, France) were used. All *in vitro* data were derived from 10 independent experiments using 20 to 30 mice in each experiment.

Isolation of bone marrow cells

Animals were sacrificed by carbon dioxide inhalation followed by cervical dislocation. The femurs and tibias were dissected out, and a cell suspension was prepared by flushing the femurs and tibias. The cell suspension was filtered through a 70-µm cell strainer (Falcon, Becton Dickinson).

Isolation of muscle cells

Muscle tissue from the tibialis anterior was dissected, bones and tendons were removed, and the excised muscles were carefully minced with a scalpel. Muscle tissue was weighed and enzymatically digested using a solution of Dispase II at 3.5 ml/g of tissue (Roche Diagnostics) with 0.2% of Collagenase A (Roche Diagnostics) supplemented with 2.5 mM $CaCl_2$ for 1.5 h at 37 °C. The cell suspension was then filtered and centrifuged at 280 × g for 10 min at room temperature before further analysis.

Cell staining and flow cytometry
Hoechst 33342 staining

Before Hoechst staining, red blood cells were lysed using an NH_4Cl buffer, following the manufacturer's recommendations (Stem Cell Technologies).

Mononuclear cells were centrifuged, pelleted and cell concentrations were adjusted to 10^6 cells/ml using PBS 1× (GIBCO BRL) at 37 °C, containing 1% of fetal calf serum (FCS; Hyclone).

Hoechst 33342 (Molecular Probes Eugene, OR, USA) was added at a final concentration of 5 µg/ml for bone marrow and blood, and 11.5 µg/ml for muscle. Cell suspensions were incubated at 37 °C for 90 min. To block the extrusion of Hoechst from cells, control experiments were performed using 100 µM of verapamil for every 10^6 cells stained with Hoechst. After washing twice, Hoechst-stained cells were re-suspended in PBS 1×, containing FCS 1%, at 37 °C.

Antibody staining

Freshly isolated cells or Hoechst-stained cells were incubated with the CD45-APC antibody or the appropriate isotype control IgG$_{2b, κ}$-APC (all from Pharmingen) for 20 min at room temperature. After washing, they were re-suspended in PBS 1×, containing 1% FCS, filtered and stained with 1 µg/ml 7-aminoactinomycin D (Sigma) to identify non-viable cells. The cells were then sorted by flow cytometry on FACS Diva (Becton Dickinson).

Phenotypic analysis

The cell suspension of muscle, marrow and blood were co-stained with CD45- and CD3, CD11b, CD19, CD31, CD34, B220, pan-NK, Gr1, TER119, Thy-1, Sca-1 and c-kit antibodies (all from Pharmingen).

Assays for hematopoietic activity
Clonogenic assay

Clonogenic assays were performed in 1% methylcellulose in Iscove's modified Dulbecco's medium (IMDM; MethoCult™ M3134, Stem Cell Technologies), including 1% penicillin-streptomycin (GIBCO BRL), 10% FCS, 10% bovine serum albumin (BSA; Sigma), 10 µg/ml insulin (Sigma), 2 mM L-glutamine (GIBCO BRL), 5 U/ml rhuman-erythropoietin (Cylag, France), murine stem cell factor (SCF; BHK cell line conditioned medium 5%), murine interleukin-3 (IL-3; WEHI-conditioned medium, 10%), 10 ng/ml rmurin-IL-6 (Abcys), 10 ng/ml rh-granulocyte colony-stimulating factor (Aventis), 5 ng/ml PEG-rhu-MGDF (Kirin, Brewery, Japan) and 450 µM α-monothioglycerol (Sigma Aldrich).

Cells were plated in 1 ml of complete medium in 35-mm bacterial-grade dishes (Greiner, Corning) as follows: $2 × 10^5$ total muscle nucleated cells; $5 × 10^4$ total marrow or blood nucleated cells and CD45$^+$ purified cells; 500 SP CD45$^+$ cells isolated from muscle; and 250 SP CD45$^+$ cells isolated from marrow. Dishes were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Colonies were counted at day 8 after plating using an inverted microscope.

Long-term culture assays

The LTC-IC frequency was established by culturing cells in a limiting dilution ($n = 4$ experiments) with weekly half-medium changes for 5 weeks as previously described [10]. Briefly, these experiments were performed in the presence of MS-5 cell layers in MyeloCult™ M5300 (Stem Cell Technologies) that were supplemented with 10µM hydrocortisone (Sigma). Cultures were incubated at 33 °C in a humidified atmosphere with 5% CO_2. At week +5, adherent and non-adherent cells from each well were combined and assayed in methylcellulose and colonies were counted at day 8.

Proliferation and differentiation assays of single SP/CD45$^+$ cells

Single cells were sorted using FACS into individual U-bottomed microwell plates in IMDM (GIBCO BRL), which included 4% FCS, 1% BSA, 5 µg/ml transferrin (Sigma) and 5 µg/ml insulin, or on an OP9 feeder in flat-bottom 96-well plates in the presence of α-MEM (GIBCO BRL), supplemented by 1% penicillin-streptomycin and 10% FCS. The culture media were supplemented in both cases with 10 µg/ml rhu-Flt3-ligand (Immunex), 10 µg/ml SCF, 10 ng/ml rm-IL-11 (R&D Systems, Minneapolis) and 10 ng/ml rm-IL-7 (R&D Systems, Minneapolis) for 14 to 28 days.

The numbers of cells were evaluated at several timepoints (3-28 days) by visual inspection or counting. The doubling rate was calculated using the following formula: Log(number of cells)/Log(2). By day 12 of the culture, cells were collected, and a phenotypic analysis was carried out. The cells were stained with anti-CD11b, B220, pan NK and/or CD11c, and CD80 antibodies. They were then analyzed with flow cytometry (FACS Sort BD) using the CellQuest Pro Software. Single-cell expansion data were obtained in nine different SP/CD45$^+$ cell-sorting experiments (marrow = 4; muscle = 5) and each clone was separately phenotyped as indicated.

Culture on OP9 cells

The OP9 mouse bone marrow stromal cell line (macrophage colony-stimulating factor (M-CSF)-deficient) was generously provided by Dr Toru Nakano (Research Institute for Microbial Diseases, Osaka University, Japan). This cell line was maintained on gelatinized 75 cm^2 flask in the OP9 growth medium: α-MEM supplemented with 1% penicillin-streptomycin and 20% FCS (Hyclone).

Results

CD45⁺ cells derived from marrow, peripheral blood and muscle differ in their phenotype

To determine whether the CD45⁺ cells isolated from marrow, peripheral blood (PB) and muscle displayed any differences in terms of phenotype, we first comparatively analyzed the phenotype of CD45⁺ cells isolated from these three tissues. As shown in Figure 1, several identical markers and several differences were noted between the three cell sources. The subpopulations of CD45⁺ cells that express CD3, B220 and CD19 (CD45⁺/CD3⁺, CD45⁺/B220⁺ and CD45⁺/CD19⁺ cells) were relatively similar between muscle and blood, whereas the percentages of these cells were twice as low in the marrow. There were also significant differences in CD45⁺/Thy1⁺ and CD45⁺/Sca1⁺ populations from blood, marrow and muscle origins (Figure 1). CD11b expression was almost identical in cells from the three sources. We cannot exclude the possibility that some cells express multiple markers of the myeloid, T-cell and B-cell lineages. The percentages of CD34⁺ and CD31⁺ cells were significantly higher in muscle and marrow than in blood-derived CD45⁺ cells. The expression of CD31 could be associated with a functional effect, since, in clonogenic assays, CD45⁺/CD31⁺ cell sorting allowed a significant enrichment of clonogenic potential in BM cells, whereas CD45⁺/CD31⁻ cells had more clonogenic activity in the muscle (data not shown).

The use of CD45⁺ and SP markers allows an enrichment of the clonogenic potential of total marrow and muscle cell fractions

The SP phenotype allows a significant enrichment of the stem cell potential from various tissues [11]. We therefore asked whether muscle, marrow and blood CD45⁺ cells would contain different percentages of SP cells. The isolation of right and left tibialis anterior muscles allowed the routine collection of 3 g of muscle per mouse with the yield of 5×10^6 cells/g of muscle tissue after enzymatic digestion. In several experiments, murine muscle was found to contain consistently higher percentages of SP cells (1-3%, $n = 10$) compared to marrow (0.2-0.8%, $n = 10$). As shown in the experiment in Figure 2, SP cells isolated from marrow had the CD45⁺ phenotype almost exclusively (99%), whereas SP cells isolated from muscle were only partially CD45⁺ (28%).

In absolute numbers, bone marrow contained more SP/CD45⁺ cells (8×10^4 to 3.2×10^5 per 2 femurs) compared to muscle (5×10^4 to 1.5×10^5 cells per 2 tibialis anterior muscles) owing to its high content of mononuclear cells ($4\text{-}5 \times 10^7$ cells for both femurs and tibias) and the high percentage of CD45⁺ cells that it harbors.

In vitro hematopoietic progenitor activity was comparatively evaluated in whole marrow, muscle and blood, and in several purified fractions. The clonogenic potential of total marrow cells was found to be 17-fold higher compared with total muscle cells and 200-fold higher compared with total blood (Figure 3A). When total nucleated cells were sorted using an anti-CD45 antibody, only the CD45⁺ cell fraction generated hematopoietic colonies in marrow and
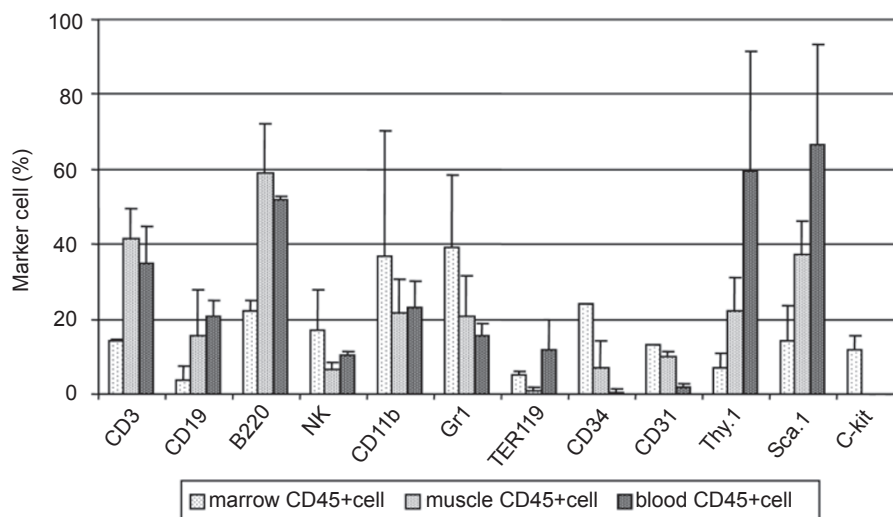

Figure 1 Comparative phenotypic analysis of purified CD45⁺ cells isolated from marrow, muscle and blood. Double-labeling experiments were performed to determine the contribution from subpopulations of lineage-specific cells to the CD45⁺ cell fraction of marrow, muscle and blood.
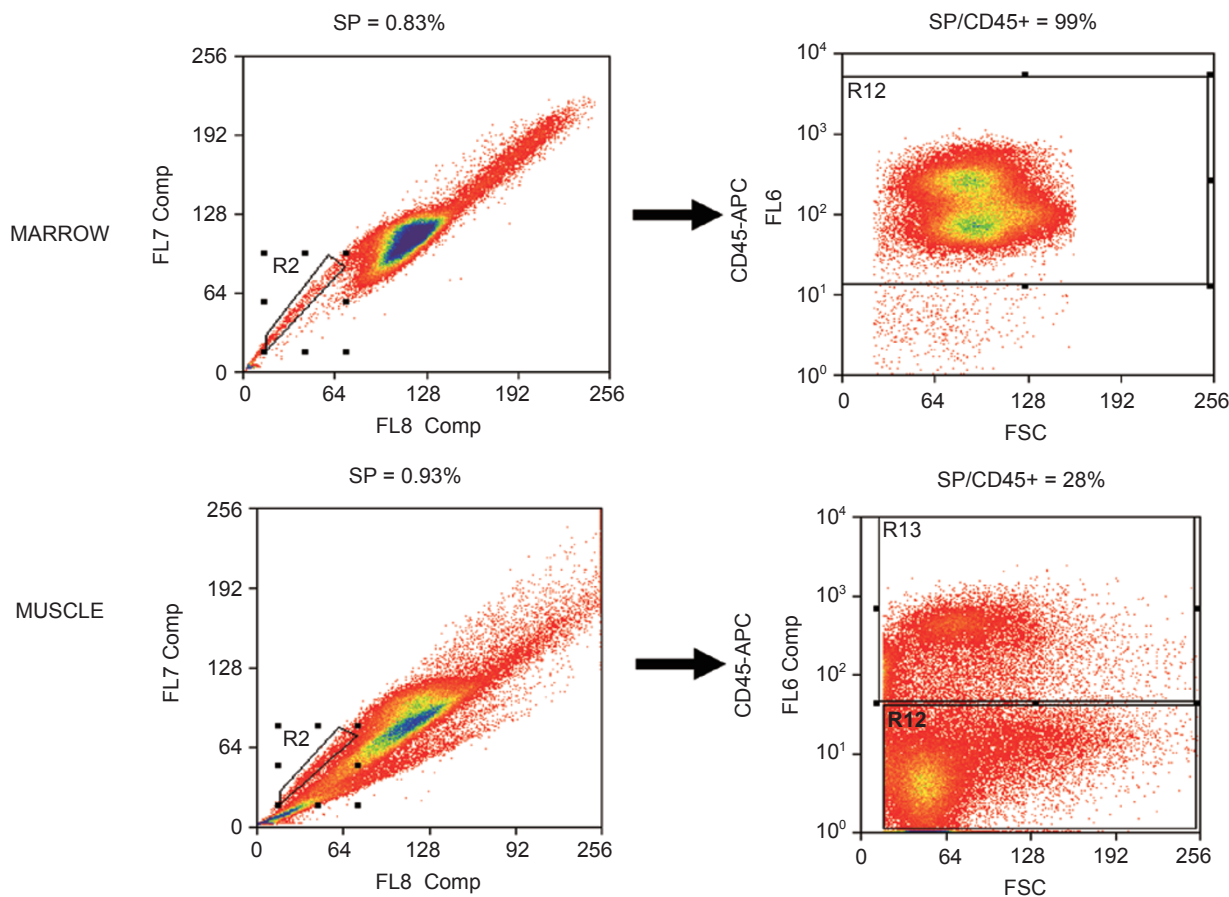
Figure 2 A representative experiment showing the approaches taken to isolate SP and SP/CD45$^+$ positive cells from muscle and marrow. Cells stained with Hoechst (5 μg/ml for marrow and 11.5 μg/ml for muscle) were incubated with an anti-CD45-APC antibody and CD45$^+$ cells were sorted in the SP cell fraction. In the experiment shown, the percentage of SP cells was 0.83% for marrow and 0.93% for muscle. In the SP fraction, CD45$^+$ cell positivity was 99% for marrow and 28% for muscle.

muscle-derived cells, and colony numbers were directly proportional to the enrichment level in CD45$^+$ cells. We noted a 10-fold enrichment of clonogenic capacity in muscle-derived cells, whereas this capacity was increased by only 1.8-fold in marrow-derived cells.

We then asked whether the use of an SP marker in the CD45$^+$ cell fraction increased hematopoietic progenitor cell activity and, to this end, we sorted the SP/CD45$^+$ cell fraction from marrow and muscle and carried out clonogenic assays. The same assays also allowed the evaluation of the hematopoietic potential that originates from CD45$^+$ cells that belong to the 'main population' (MP) after incubation with Hoechst 33342. As can be seen in Figure 3B, the use of an SP marker, in addition to CD45, allowed a major enrichment of clonogenic activity in both marrow and muscle-derived mononuclear cell fractions. Indeed, we observed a 60-fold enrichment of clonogenic potential

in marrow and a 360-fold enrichment in muscle compared with unseparated cell populations. By contrast, in the MP CD45$^+$ subpopulation no clonogenic activity was detected (Figure 3B).

LTC-IC frequency of SP/CD45$^+$ cells obtained from skeletal muscle

To determine the *in vitro* hematopoietic stem cell potential of muscle SP/CD45$^+$ cells, we analyzed the long-term culture initiating capacity of these cells and measured the frequency of LTC-ICs using limiting dilution assays based on previously described standard techniques [10]. As shown in Figure 4, in the marrow-derived SP/CD45$^+$ fraction, the frequency of LTC-ICs was 1/100, whereas in the muscle-derived SP/CD45$^+$ fraction, this frequency was 1/640. In absolute numbers and depending on the efficiency of cell recovery in different experiments, this would result in
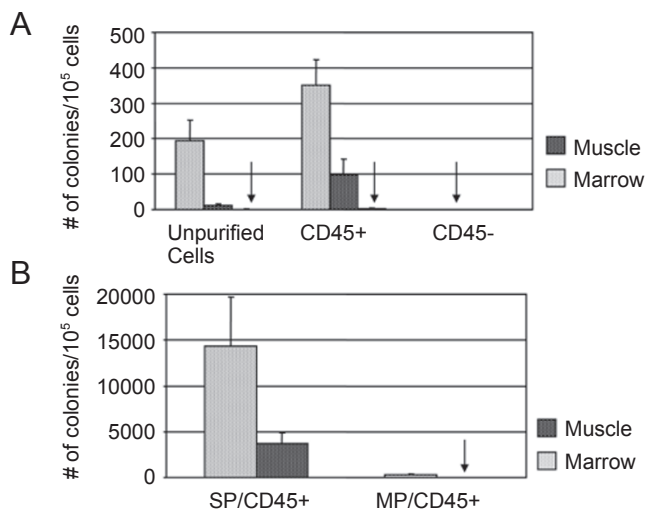
Figure 3 Clonogenic potential of unfractionated and purified cells isolated from marrow, muscle and blood. **(A)** Comparative analysis of the clonogenic potential of total marrow, muscle and blood cells and the enhancement of this potential using $CD45^+$ cells. Blood $CD45^+$ cells could not generate any clonogenic activity (as indicated by arrows). No enhancement could be obtained in $CD45^+$ cells from blood and in CD45- cells from any cell sources (see text). **(B)** Evidence of a major enrichment of the clonogenic potential using $SP/CD45^+$ cells from muscle compared with a lack of enrichment of the clonogenic activity in the $CD45^+$ cells of the main population. The arrow indicates the absence of clonogenic cells from muscle-derived $CD45^+$ cells from the main population.

800-3200 LTC-ICs for marrow (2 femurs and 2 tibias) and 78-234 LTC-ICs for muscle (2 tibialis anterior muscles).

To determine qualitatively the progenitor activity in this fraction, we measured the clonogenic distribution of LTC-IC-derived progenitors as a function of the number of cells that were used to initiate the long-term cultures. The percentages of wells containing 1-10, 10-100 and >100 colonies were distributed relatively homogeneously in cultures that were started with different numbers of marrow cells, whereas the distribution was more hetero-geneous in the muscle-derived fraction, with evidence of increased clonogenic activity that had no relationship with the numbers used to initiate the long-term cultures (Figure 5). Thus, these results demonstrate that muscle tissue con-tains primitive stem cells purified by $SP/CD45^+$ markers and these cells can be quantified using LTC-IC assays. The distribution frequency of the LTC-IC-derived progenitors suggests however that this subpopulation is heterogeneous in terms of its stem cell potential (Figure 5).

Amplification and multilineage differentiation ability of SP/CD45⁺ cells that originate from muscle versus marrow

In order to determine whether $SP/CD45^+$ cells that are isolated from muscle, marrow and blood have different functional properties, we performed single-cell-sorting experiments in the presence of early-acting cytokines (SCF,
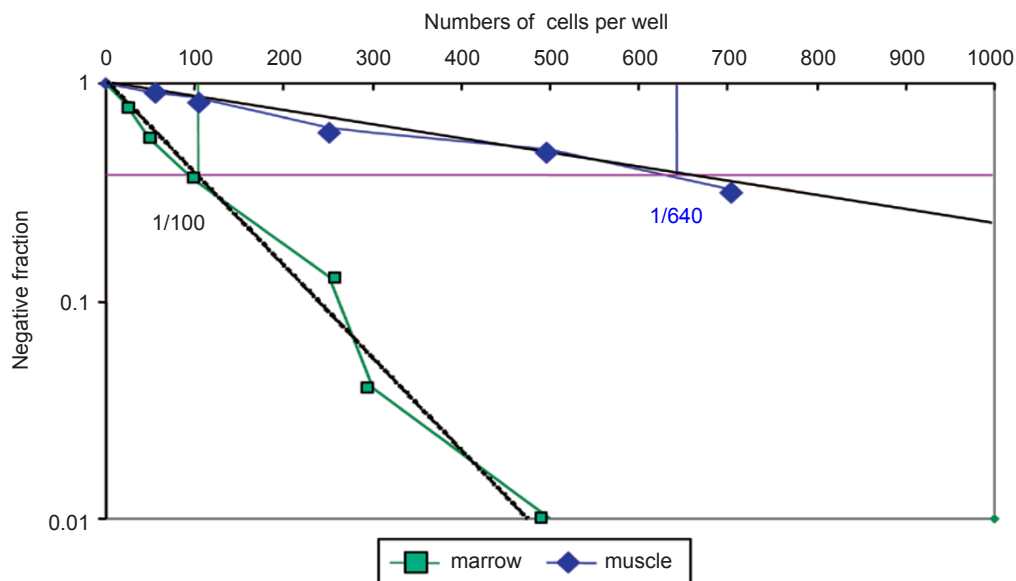

Figure 4 Evaluation of long-term culture initiating cell (LTC-IC) frequencies generated by marrow- and muscle-derived $SP/CD45^+$ cells. $SP/CD45^+$cells were sorted from marrow and muscle and were cultured on limiting dilution assays of murine MS-5 stroma 96-well plates at 33 °C for 5 weeks with weekly half-medium changes. At week +5, the content of each well was transferred to a single dish containing methylcellulose with growth factors. The numbers of LTC-IC-derived clonogenic cells were then determined by counting the numbers of colonies at day +7. By determining the numbers of positive (growth) and negative (no growth) wells, the LTC-IC frequency was calculated using Poisson's distribution.
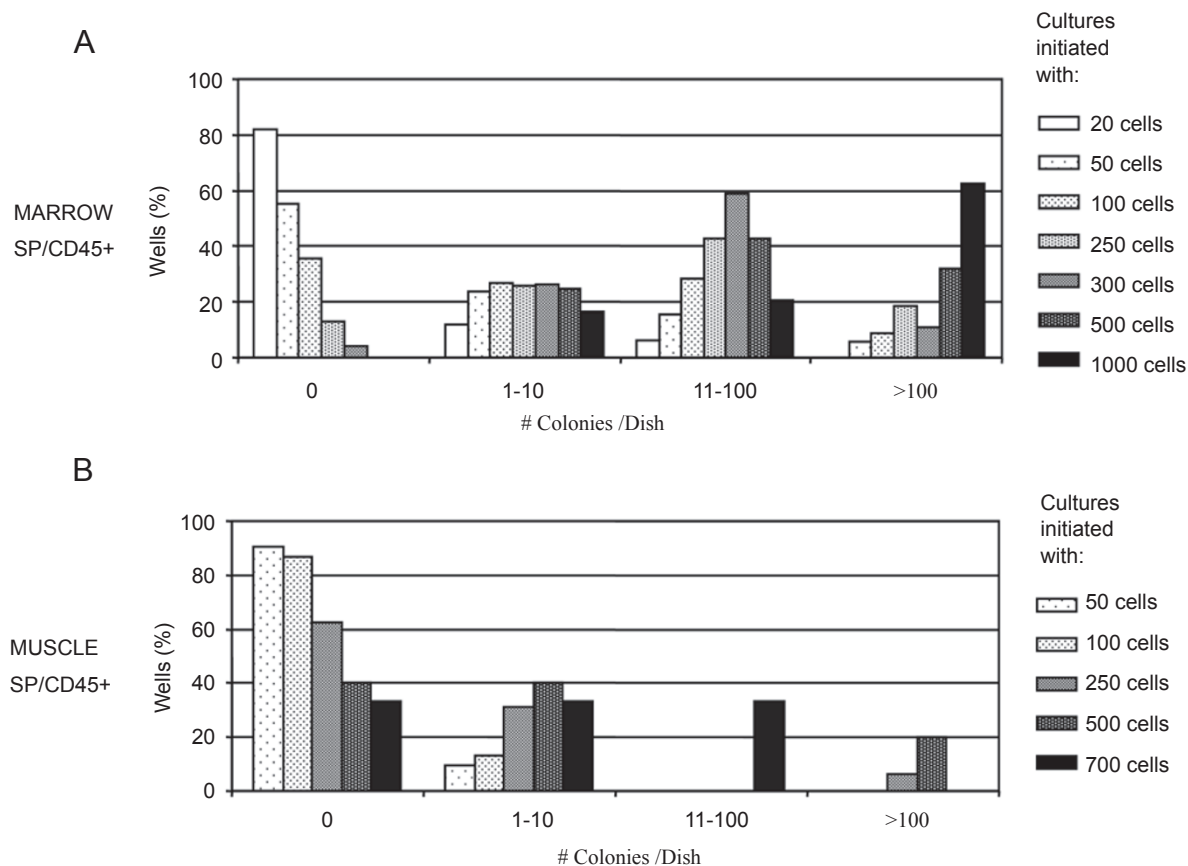
A

B

Figure 5 Comparative analysis of the clonogenic distribution of LTC-IC (long-term culture initiating cell)-derived progenitors that originate from bone marrow and muscle. In limiting dilution assays used to calculate the LTC-IC frequencies, the numbers of positive wells were analyzed in terms of their content in order to determine the clonogenic distribution. As shown, with increasing numbers of cells plated in LTC-IC assays (20-1000 cells), the numbers of colonies obtained in methylcellulose increased proportionally in cultures that were started with marrow-derived SP/CD45$^+$ cells. On the other hand, there was no clear-cut relationship between the clonogenic cell yield and the numbers of muscle-derived SP/CD45$^+$ cells seeded in long-term cultures, which suggests a heterogeneity of stem cell potential in this population. In order to unravel this potential, single-cell-sorting experiments were performed (see text, Table 1 and Figure 6).

l-Flt3, IL-7 and IL-11) with the goal of individually testing the amplification and multilineage differentiation potential of the FACS-sorted cells. The same cells were also cultured in the presence of OP9 cell lines, which support the growth and differentiation of hematopoietic cells from murine ES cells [12]. As shown in Table 1, single SP/CD45$^+$ cells that were sorted from marrow ($n = 4$ experiments) and analyzed between day 4 and day 28 generated 109 out of 768 wells that scored positively on the basis of cell growth. By contrast, 42 wells out of 1248 muscle SP/CD45$^+$ cells ($n = 5$ experiments) scored positively under the same conditions. Thus, the cloning efficiency of muscle-derived single SP/CD45$^+$ cells was found to be one-quarter of that observed in marrow-derived SP/CD45$^+$ cell populations. The same analysis was also performed in SP/CD45$^+$ cells that were

isolated from PB. The cloning efficiency of blood-derived SP/CD45$^+$ cells was extremely low (one out of 191 wells were positive) compared with marrow-derived (one out of seven wells were positive) and muscle-derived (one out of 30 wells were positive) SP/CD45$^+$ cells (Table 1).

We then asked whether the unique FACS-sorted SP/CD45$^+$ cells cultured in the presence of early-acting cytokines had similar potentials for undergoing amplification and multilineage differentiation. To this end, individual cells that originate from marrow, muscle and blood were visually monitored and counted for up to 21 days. The absolute numbers of cells were determined at different time periods (days 4-28) and, whenever possible, a phenotypic analysis was performed in individual wells.

As shown in Table 1, marrow-derived individual SP/

Table 1 Cloning frequency and amplification potential of single SP/CD45+ cell from marrow, muscle and blood

Tissue	Culture Conditions	Number of well	Positive wells	Cloning efficiency	Fold-increase (mean)	Doubling rate
Marrow	cytokines	768	109	1/7	3 - 500[b] (157)	1.6 - 9
					40 - 7920[d] (2349)	8.3 - 12.9
	cytokines + OP9	216	32	1/6.7	300 - 470150[c] (87785)	8.2 - 18.8
Muscle	cytokines	1248	42	1/30	7 - 150[b] (82)	2.8 - 7.2
					40 - 720[d] (380)	5.3 - 9.5
	cytokines + OP9	534	21	1/25	20000 - 328900[c] (148112)	14.3 - 18.3
Blood	cytokines	955	5	1/191	7 - 50[a] (29)	2.8 - 5.6

[a] cell numbers determined at day 7

[b] cell numbers determined at day 11

[c] cell numbers determined at day 12

[d] cell numbers determined at day 16

CD45^{+} cells underwent a major amplification at day 16 (mean 2349-fold, range 40-7920), with a doubling rate that varied between 8.3 and 12.9. Under the same conditions, the SP/CD45^{+} cells isolated from muscle also exhibited significant amplification potentials in absolute numbers, despite the lower cloning efficiency, as the mean amplification rate was 380-fold (range 40-720), with a doubling rate that varied between 5.3 and 9.5. These findings establish that muscle-derived SP/CD45^{+} cells have a lower amplification potential compared with marrow-derived cells. Interestingly, when the same experiments were performed in the presence of OP9 cell lines and cytokines, there was an enhancement of the amplification potential and the doubling rate in both marrow and muscle-derived SP/CD45^{+} cells without any modification of their cloning efficiencies (Table 1). When blood-derived SP/CD45^{+} cells were cultured under the same conditions, we found a 7- to 50-fold amplification rate and a doubling rate that varied between 2.8 and 5.6 (Table 1). However, no growth could be obtained after day 7 of culture. Overall, these results clearly show that SP/CD45^{+} cells that have an amplification potential exist in the murine skeletal muscle, but their frequency is lower than their marrow-derived counterparts.

In order to determine the stem cell potential of these cell populations, we tested their multilineage differentiation ability. Cultures amplified from single cells were tested using myeloid, B, NK and dendritic cell differentiation markers. According to the presence of CD11b, B220 and NK cell markers, single-cell-derived clones were classified as unipotent (single marker), bipotent and tripotent. The multilineage differentiation ability of muscle-derived SP/CD45^{+} cells was twice as high as that of marrow-derived cells as indicated by the percentages of tripotent cells (Figure 6). Muscle tissue also contained cell populations with single

or bilineage differentiation ability in percentages that were comparable to those found in bone marrow-derived cells. These results demonstrate that muscle tissue contains genuine stem cells with multilineage differentiation ability.

Discussion

Previous studies have suggested that muscle-derived hematopoietic stem cell activity is low compared with bone marrow-derived cells of the same phenotype (c-kit[+]/SP^{+} cells). In these experiments, c-kit expression was found to be lower in muscle-derived cells, and this was not due to the protease treatment of muscle cells during the digestion of this tissue [14]. In our experiments, to comparatively examine the hematopoietic stem cell activity in both tissues we chose the SP/CD45^{+} markers as we were not able to identify c-kit^{+} cells in the muscle tissue after digestion by dispase and collagenase [3]. We first compared CD45^{+} cells from bone marrow, blood and muscle tissues by double marker analyses using myeloid, lymphoid and endothelial differentiation markers. Our results demonstrated significant differences in the three sources of CD45^{+} cells by the use of Sca1 and Thy1 (which appeared to be highly expressed in CD45^{+} blood cells) and CD11b and Gr1 (highly expressed in CD45^{+} marrow cells), but none of the markers used appeared to be highly characteristic of muscle-derived CD45^{+} cells. *In vitro*, the clonogenic efficiency of muscle CD45^{+} cells was enriched by the use of SP/CD45^{+} cell populations. We therefore chose this fraction for the quantification of LTC potential and LTC-IC frequencies. The comparative analysis of marrow and muscle-resident SP/CD45^{+} cells showed for the first time, to our knowledge, that muscle tissue contains significant LTC-IC potential, which appears to be one out of 640; in
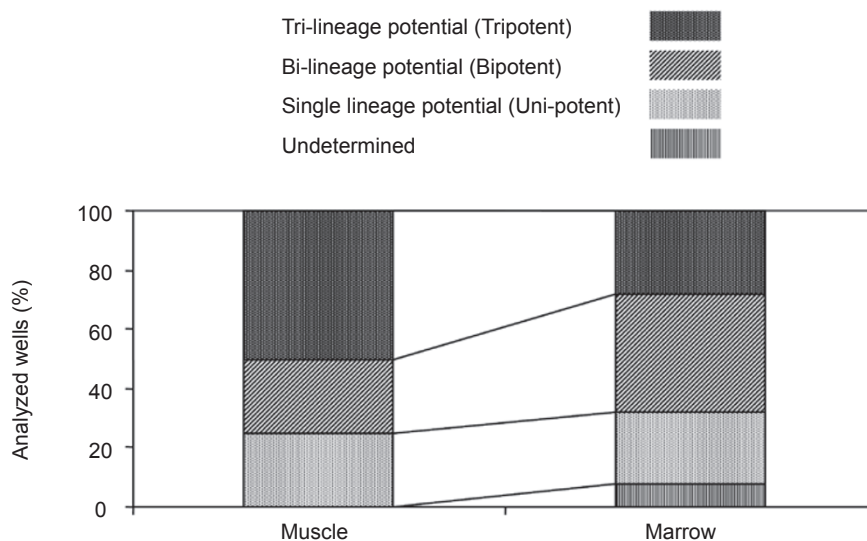
Figure 6 Multipotentiality of single SP CD45$^+$ cells isolated from marrow and muscle. Single SP/CD45$^+$ cells were cultured under the conditions described in the 'Materials and Methods'. At different time points (see Table 1), the wells with one, two or three differentiation lineage markers were classified as unipotent, bipotent and tripotent, respectively, corresponding to the detection of CD11b$^+$, NK$^+$ and CD19$^+$ cells. Muscle-derived SP/CD45$^+$ cells exhibited evidence of significant trilineage differentiation capacity.

other words, it is about one-sixth of the frequency observed for marrow-derived SP/CD45$^+$ cell population. A previous study by Geiger *et al*. [8] also confirmed the hematopoietic stem cell potential in muscle by using a cobblestone area-forming cell (CAFC) assay. Compared with the results reported here, we found CAFC activity in the Sca1-intermediate, CD31$^+$ and CD45$^+$ cells, but did not evaluate the SP/CD45$^+$ cell fraction. Nevertheless, these results confirm the fact that the presence of CD45$^+$ is essential for both clonogenic and stem activity of muscle-resident stem cells, the origin of which remains undetermined at this time. Interestingly, in our experiments, the analysis of clonogenic distribution in the muscle fraction indicated a major difference between the SP/CD45$^+$ cells from the muscle and marrow origins, with the former population exhibiting a heterogeneous pattern. This heterogeneity prompted us to analyze both populations at the clonal level using single-cell purification experiments. To this end, we compared the cloning efficiency, the amplification potential and the multilineage differentiation ability of single cells derived from both fractions, using a combination of cytokines to allow lymphoid and myeloid differentiation as previously described [13]. Under these conditions, the cloning efficiency of SP/CD45$^+$ cells was found to be one in seven in the marrow-derived cells and one in 30 in the muscle-derived cells. Interestingly, this frequency was not changed in both tissues when the single cells were grown in the presence of the OP-9 stroma. However, the OP-9 stroma

in combination with cytokines induced a major amplification of both marrow and muscle-derived SP/CD45$^+$ cells, suggesting that under the appropriate microenvironmental conditions the muscle-derived single SP/CD45$^+$ cells could exhibit a major amplification potential. The blood-derived SP/CD45$^+$ cells had a very low cloning efficiency and did not show any significant amplification potential since no growth was obtained after 7 days. Most importantly, phenotypic analysis of individual wells of marrow and muscle origins showed the multilineage differentiation ability of both marrow- and muscle-derived single SP/CD45$^+$ cells, with evidence of higher multilineage differentiation ability in muscle-derived wells.

Overall, our experiments suggest that the altered hematopoietic stem cell potential of muscle-derived cells is not due to a global reduction of this potential as previously suggested [14], but to the heterogeneity of this population. Indeed, under the appropriate conditions, this population exhibited significant stem cell activity *in vitro* (as indicated by the multilineage differentiation ability in response to cytokines and microenvironmental signals). This hypothesis is also strengthened by our *in vitro* LTC experiments in which the LTC-IC frequency is lower in SP/CD45$^+$ cells of muscle origin than in those of marrow origin, with heterogeneous clonogenic distribution on a well-to-well basis. In single-cell experiments, we demonstrated that the SP/CD45$^+$ cells obtained from muscle have a multilineage differentiation ability that is similar to their marrow-derived counterparts.

Why the murine muscle is a site of hematopoietic stem cell potential is currently undetermined. Although it has been suggested that these cells are of marrow origin [4, 5], there is currently no evidence to demonstrate the pre- or post-natal origin of these cells. The real *in vivo* hematopoietic potential of these cells should be evaluated in the future in single-cell transplantation experiments.

Acknowledgments

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(**Supplementary information** is linked to the online version of the paper on the Cell Research website.)