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Identification of a putative pathway for the muscle homing of stem cells in a muscular dystrophy model

Yvan Torrente,[1,4] Geoffrey Camirand,[3] Federica Pisati,[1] Marzia Belicchi,[1] Barbara Rossi,[5] Fabio Colombo,[2] Mosthapha El Fahime,[3] Nicolas J. Caron,[3] Andrew C. Issekutz,[7] Gabriela Constantin,[5] Jacques P. Tremblay,[3] and Nereo Bresolin[1,6]

[1]Department of Neurological Sciences, Stem Cell Laboratory, and [2]Institute of Nuclear Medicine, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Ospedale Maggiore Policlinico, 20122 Milan, Italy
[3]Université Laval, Unité de Génétique Humaine, Centre Hospitalier de l'Universite Laval, Ste-Foy, G1K 7P4 Quebec, Canada
[4]Centro Dino Ferrari, Department of Neurological Sciences, University of Milan, 20122 Milan, Italy
[5]Department of Pathology, Division of General Pathology, University of Verona, 37129 Verona, Italy
[6]IRCCS Eugenio Medea, 20038 Bosisio Parini, Italy
[7]Department of Pediatrics, Pathology, and Microbiology-Immunology, Dalhousie University, Nova Scotia, Canada B3H 4H6

Attempts to repair muscle damage in Duchenne muscular dystrophy (DMD) by transplanting skeletal myoblasts directly into muscles are faced with the problem of the limited migration of these cells in the muscles. The delivery of myogenic stem cells to the sites of muscle lesions via the systemic circulation is a potential alternative approach to treat this disease. Muscle-derived stem cells (MDSCs) were obtained by a MACS® multisort method. Clones of MDSCs, which were Sca-1$^+$/CD34$^-$/L-selectin$^+$, were found to adhere firmly to the endothelium of *mdx* dystrophic muscles after i.v. or i.m. injections. The subpopulation of Sca-1$^+$/CD34$^-$ MDSCs expressing L-selectin was called homing MDSCs (HMDSCs). Treatment of HMDSCs with antibodies against L-selectin prevented adhesion to the muscle endothelium. Importantly, we found that vascular endothelium from striate muscle of young *mdx* mice expresses mucosal addressin cell adhesion molecule-1 (MAdCAM-1), a ligand for L-selectin. Our results showed for the first time that the expression of the adhesion molecule L-selectin is important for muscle homing of MDSCs. This discovery will aid in the improvement of a potential therapy for muscular dystrophy based on the systemic delivery of MDSCs.

Introduction

The absence of dystrophin in the membrane-associated cytoskeleton of muscle fibers leads to their degeneration, resulting in a lethal muscle wasting disease known as Duchenne muscular dystrophy (DMD) (Hoffman et al., 1987; Emery, 1989). The number of satellite cells (myogenic precursors) in dystrophic muscles decreases over repeated cycles of degeneration and regeneration, and eventually there are not enough cells to allow adequate muscle regeneration. The skeletal muscle is then replaced by connective tissue, leading to a progressive muscle weakness.

Populations of pluripotent stem cells were obtained from muscles using different procedures. A side population (SP) was initially obtained from muscles by FACS® on the basis of Hoechst dye exclusion (Gussoni et al., 1999; Jackson et al., 1999). Muscle-derived stem cells (MDSCs) were also obtained by a series of preplatings and shown to express Sca-1 and CD34 (Lee et al., 2000; Deasy et al., 2001; Torrente et al., 2001). These MDSCs have the capacity to differentiate into all major blood lineages in vitro (Torrente et al., 2001). Moreover, bone marrow restoration was observed after injection of muscle SP cells (a stem cell population obtained by FACS®) into the tail vein of lethally irradiated mice (Jackson et al., 1999). Of particular significance is the observation that transplanted SP cells isolated from bone marrow or muscle also actively participated in myogenic regeneration. MDSCs and satellite cells are distinct cell populations, as demonstrated by the normal numbers of MDSCs and the complete absence of satellite cells in Pax7 (gene specifically

Address correspondence to Yvan Torrente, Department of Neurological Sciences, University of Milan, Padiglione Ponti, Ospedale Policlinico, via Francesco Sforza 35, 20122 Milan, Italy. Tel.: 39-02-55033874. Fax: 39-02-50320430. email: torrenteyvan@hotmail.com

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Abbreviations used in this paper: β-gal, β-galactosidase; DMD, Duchenne muscular dystrophy; HMDSC, homing MDSC; MAdCAM-1, mucosal addressin cell adhesion molecule-1; MDSC, muscle-derived stem cells; MyHC, myosin heavy chain; SP, side population; TA, tibialis anterior.

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expressed in myoblasts derived from satellite cells) mutant muscles (Seale et al., 2000).

However, the relationship between MDSCs and the mechanisms underlying the muscle regeneration are still poorly understood: do they remain as a quiescent pool or do they contribute to form skeletal muscle fibers after extensive tissue degeneration? Systemic transplantation of bone marrow–derived stem cells or even of MDSCs had a very limited impact on muscle cell replacement and did not improve murine muscular dystrophy (Ferrari et al., 2001; Torrente et al., 2001). This might be explained by poor recruitment of bone marrow–derived stem cells and MDSCs to the dystrophic muscle. An understanding of the nature of the factors responsible for stem cell homing to muscles will be invaluable in attempts to improve systemic delivery of stem cells for muscle diseases.

In this context, our attention was focused on the expression of adhesion molecules involved in muscle homing by MDSCs. MACS® multisort columns were used to enrich Sca-1$^+$/CD34$^-$ MDSCs. We identified a clonable subset of MDSCs expressing L-selectin, an adhesion molecule critical for transendothelial migration of the blood- and bone marrow–derived cells. This subset of MDSCs will be referred to as homing MDSCs (HMDSCs). Using intravital microscopy, we showed that these HMDSCs adhered firmly to the endothelium of *mdx* muscle microvessels after i.v. or i.m. injections. Treatment of HMDSCs with an antibody against L-selectin prevented their adhesion to the blood vessels. Intravenous injections of HMDSCs, obtained from transgenic newborn mice carrying a LacZ reporter gene under the desmin promoter, produced β-galactosidase (β-gal)– and dystrophin-positive fibers in many muscles. The discovery of the mechanism involved in the muscle homing of MDSCs will aid in the improvement of a potential therapy for muscular dystrophy based on the systemic delivery of such stem cells.

Results

Ability of injected MDSCs to induce chimera in *mdx* mice

To isolate the MDSCs, we used a previously described approach that had proven successful for the isolation of stem and progenitor cells from the muscle, namely preplatings (Qu et al., 1998; Torrente et al., 2001). 500,000 MDSCs from male F1 Balb/c-C57BL10J mice (H2db) were injected intramuscularly in 10 female *mdx*/*mdx* mice (H2b). Using donor cells from an F1 donor, thus expressing the host and the donor major histocompatibility complexes (H2db), prevented a graft versus host disease. The presence of donor-derived cells in blood and bone marrow was still detectable by FACS® analysis for H2d. The donor cells were also evident by PCR for the Y chromosome in the injected tibialis anterior (TA) muscle, controlateral hind limb muscles, blood, and bone marrow, but not in sample organs such as the liver, spleen, lung, and brain (Fig. 1 b). The animals seemed in good health throughout the experimental period (3 wk). At 1 and 3 wk after i.m. injection of MDSCs, the flow cytometric analysis of peripheral blood nucleated cells revealed the presence of <1% positive H2d (donor) cells. A similar percentage (0.1–0.5%) was also detected in the bone marrow tissues after 3 wk. Recipient blood and bone marrow tissues were analyzed for the presence of donor MDSCs by double staining for H2d and one of the following markers: Sca-1, Flk-1, CD34, CD90, and CD45. 3 wk after MDSC i.m. engraftment, whole-body perfusion with saline solution was done to exclude contamination of circulating cells in tissues. Muscle tissues were dissociated mechanically and enzymatically and analyzed by FACS® for H2d donor antigen. We observed <5% of donor cells in the controlateral hind limb muscle tissues, and the cells expressing H2d also expressed the stem cell markers Sca-1 and CD34 (Fig. 1 c). This analysis indicated a selective engraftment by donor cells of mesodermal origin.

To determine whether donor MDSCs present in the bone marrow tissues of *mdx* are differentiated and able to induce tolerance, we grafted the skin of F1 Balb/c-C57BL/10J male (H2db) into 6 of the 10 female *mdx*/*mdx* mice (H2b) 2 wk after the i.m injection of MDSCs. All the recipient *mdx*/*mdx* mice rejected the skin grafts within 7 d. FACS® analysis showed that chimeric donor H2db cells extracted from the host muscles, blood, and bone marrow were CD45 negative. These data indicate that MDSCs in the chimeric bone marrow of *mdx*/*mdx* mice maintained a stem cell state (Sca-1$^+$, CD34$^-$, and lineage negative) and did not express hematopoietic activity.

Adhesion of one subpopulation of MDSCs to muscle microcirculation

MDSCs (obtained by a series of six preplatings) contain cells capable of adhesion to muscle blood vessels, migration, engraftment, and myogenic differentiation after intravascular injection in mice (Torrente et al., 2001). Flow cytometric analysis for Sca-1 and CD34 of MDSCs showed different subpopulations. One subpopulation comprising 69% of the MDSCs was positive for both Sca-1 and CD34. A second subpopulation containing 24% of the MDSCs was Sca-1 positive but CD34 negative, and a third subpopulation was only positive for CD34 (7%) (Fig. 1 a).

To identify which cells within the MDSCs were capable of muscle homing, the MDSCs were separated into subpopulations (i.e., Sca-1$^-$/CD34$^+$, Sca-1$^+$/CD34$^+$, and Sca-1$^+$/CD34$^-$) by immunomagnetic selection (MACS® multisort). We then tested the adhesion to muscle blood vessels of these three subpopulations of MDSCs. Boluses of 5×10^5 fluorescently labeled cells were injected into the tail vein or into the quadriceps muscle, and their adhesion to the pectoral muscle vessels was recorded. After i.v. or i.m. injections, no vascular interaction was observed for the Sca-1$^-$CD34$^+$ subpopulation. A few Sca-1$^+$/CD34$^+$ cells interacted with muscle capillaries after i.v. injection but not after i.m. injection. The Sca-1$^+$/CD34$^-$ cells were clearly distinguishable from the other two MDSC subpopulations by their significant migration within injected quadriceps after single injection (Fig. 2, c and d). The β-gal staining of muscles injected with Sca-1$^+$/CD34$^-$ MDSCs derived from ROSA26 mice confirmed the presence of donor cells spread within myofibers and near muscle capillaries and arterioles (Fig. 2, e–g). Moreover, after i.m. injections, these cells were able to migrate from the injected muscle tissue to the blood stream and then adhered to the endothelial lining of several distant muscles (Fig. 2, h–m).

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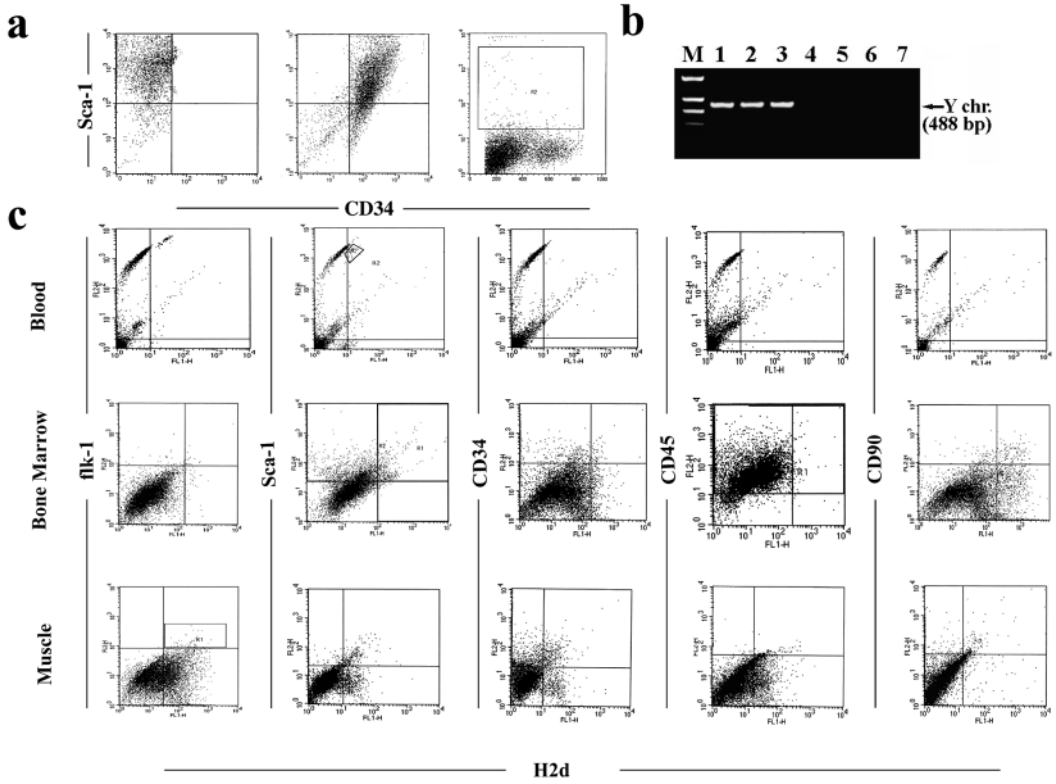

Figure 1. **FACscan® immunophenotyping of the muscle-derived cells isolated using MACS® columns.** The MDSCs obtained by six preplatings were tested for CD34 and Sca-1 antigen expression. Three distinct populations of MDSCs were obtained: Sca-1$^+$/CD34$^-$ (a, first screen), Sca-1$^+$/CD34$^+$ (a, second screen), and Sca-1$^-$/CD34$^+$ (a, third screen). In b, the donor cells were detected by PCR for the Y chromosome in the hind limb muscles (lane 1), blood (lane 2), and bone marrow (lane 3), but not in samples from organs such as the liver (lane 4), spleen (lane 5), lung (lane 6), and brain (lane 7). 3 wk after i.m. transplantation of H2d unselected MDSCs in *mdx* mice (H2b), engrafted tissues were analyzed by FACS® for the presence of positive H2d (donor) cells (c). The positive H2d cells from the blood tissue (boxed region indicated in the second quadrant of the blood row) of the injected animals represent ~0.1% of the total bloodstream. A similar percentage (~1%) of donor cells was also detected in the bone marrow tissues after 3 wk (c, bone marrow row). We observed <5% donor cells in the controlateral hind limb muscles. The H2d expression in the bloodstream correlates with the expression of Flk-1 and stem cell marker Sca-1, whereas in the bone marrow, donor cells coexpressed the CD90 (12%) and Sca-1 (85%) antigens (c). The muscle tissue also contained some donor cells positive for Flk-1, CD34, and Sca-1 markers. A few donor cells (H2d positive) were also positive for CD34 (45%), Sca-1 (20%), or Flk1 (3%), as indicated by double immunostaining.

Bone marrow homing and reconstitution after lethal irradiation by injection of bone marrow cells and Sca1$^+$/CD34$^-$ MDSCs

To assess the bone marrow homing and hematopoietic activity of Sca-1$^+$/CD34$^-$ MDSCs, we injected them into lethally irradiated *mdx*/*mdx* mice. Mice transplanted only with Sca-1$^+$/CD34$^-$ MDSCs in initial experiments did not survive, perhaps as consequence of the reduced number of injected cells or because of their reduced expansion capacity. However, as expected, the transplantation in control mice of bone marrow cells (derived from mice of the host strain) alone provided a sufficient number of hematopoietic stem cells to rescue the mice from lethal irradiation. This result permitted us in a follow-up experiment to measure the ability of donor Sca-1$^+$/CD34$^-$ MDSCs to home and repopulate the host bone marrow tissue when injected in combination with a known quantity of host bone marrow cells, as a competitive population, as previously done (Jackson et al., 1999). Thus, 50×10^5 MDSCs were prepared from the muscles of donor Balb/c mice (H-2d), mixed with 1×10^7 bone marrow–derived cells from host C57BL10J *mdx*/*mdx* mice (H-2b), and transplanted into lethally irradiated *mdx*/*mdx* mice. At 6, 9, and 19 wk after transplantation, peripheral blood was drawn from the recipients and analyzed by antibody staining and flow cytometry for the presence of donor (H-2d) B cells (B220 positive), T cells (CD90 positive), and granulocytes/macrophages (Gr-1$^+$/Mac-1$^+$). Transplanted animals had 3–15% of their white blood cells, which were H-2d positive (Table I). After 19 wk, donor cells were present at high levels in each of the lymphoid and myeloid lineages, and the proportions of B cells, T cells, and granulocytes/macrophages derived from donor Sca-1$^+$/CD34$^-$ MDSCs were 2, 13, and 66%, respectively. The overall distribution of B, T, and myeloid cells in this mouse was normal. To confirm the self-renewal capacity of MDSCs, a repopulation of lymphoid and myeloid cell compartments in secondary recipients was also performed. In this experiment, the bone marrow from the rescued irradiated *mdx*/*mdx* mouse with the highest level of H-2d circulating cells was transplanted at 8×10^5 mononucleated cells per mouse into each of five lethally irradiated *mdx*/*mdx* recipients. After 6 wk, the peripheral blood was analyzed by flow cytometry. The bone marrow from this mouse rescued all five secondary recipients from lethal irradiation and contributed appreciably to the B, T, and myeloid cell compartments. The mean percentage of

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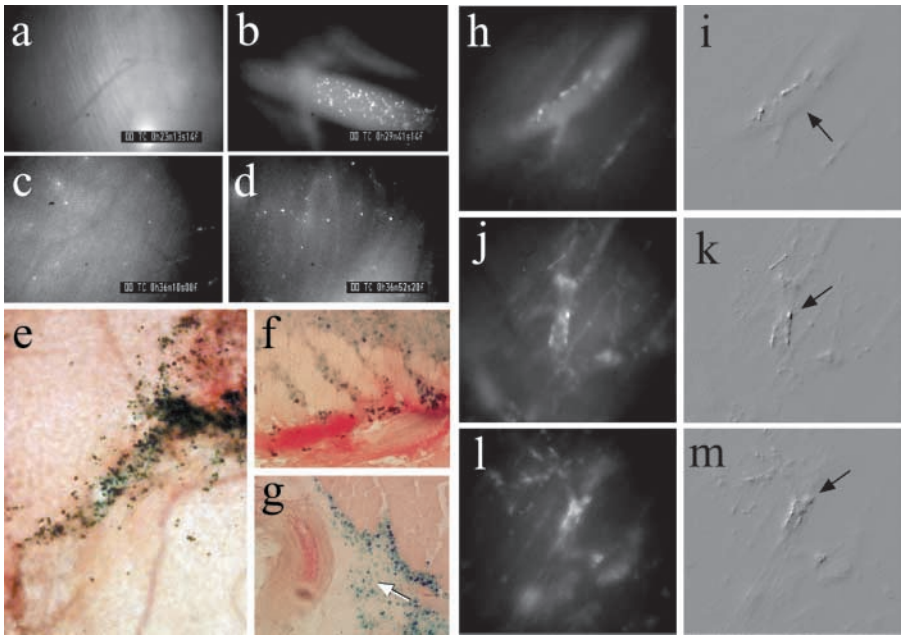

Figure 2. **We tested the adhesion of the Sca-1$^+$/CD34$^-$/L-selectin$^+$ positive cells (HMDSCs) to muscle blood vessels by intravital microscopy.** Boluses of 5×10^5 fluorescently (BCECF) labeled cells were injected into the quadriceps muscle, and their adhesion to the pectoral muscle vessels was recorded. To improve contrast between the intra- and extravascular compartments, the animals were injected intravenously with a low dose of FITC–dextran. The time course study, i.e., before (a), during (b), and after (c and d) i.m. injections, indicates a significant migration of the HMDSCs within the injected muscle. β-Gal staining of ROSA26-derived HMDSCs showed the presence of injected cells along the injection site (e), near myofibers, bleeding vessels (f), and arterioles (g). Moreover, after i.m. injections in quadriceps, the HMDSCs were able to migrate to the blood stream and then adhered to the endothelial lining of several distant muscles, such as pectoralis (h–m). Arrows in i, k, and m indicate migrated BCECF-labeled cells (bright dots) in the perivascular space of a muscle venule 1 h after injection.

H-2d$^+$ cells in the peripheral blood of these mice was 2%, and there were lower percentages of B (1.5%) and T (2%) cells and granulocytes/macrophages (11%) than in the original recipients (Table I). These data are in accordance with the results previously demonstrated by Jackson et al. (1999).

Identification of adhesion molecules involved in muscle homing

We then attempted to identify the adhesion molecules present on the Sca-1$^+$/CD34$^-$ MDSCs that may be responsible for their attachment to the blood vessels. The presence of several candidate adhesion molecules was thus investigated by FACS® analysis. No expression of E-selectin, P-selectin, VLA-4, LFA-1, PFGL-1, or ICAM-1 was observed on MDSCs. However, 30% of the Sca-1$^+$/CD34$^-$ MDSCs expressed L-selectin. The other two subpopulations of MDSCs (i.e., Sca-1$^+$/CD34$^+$ and Sca-1$^-$/CD34$^+$) did not express L-selectin.

To verify whether L-selectin was involved in the adhesion of MDSCs to blood vessels, we treated Sca-1$^+$/CD34$^-$ MDSCs with antibodies to L-selectin before their i.v. injection. This treatment reduced >70% of the adhesion of these cells to the muscle blood vessels. This demonstrated that L-selectin is involved in the migration of MDSCs to the muscles. Adhesion fractions of injected MDSCs were determined by counting the number of interacting cells in each muscle vessel per number of cells that passed through the same vessel during an injection. The subpopulation of MDSCs (Sca-1$^+$/CD34$^-$/L-selectin$^+$) capable of adhesion to muscle blood vessels represents <3% of all MDSCs and will thus be

Table I. **Multilineage engraftment in *mdx/mdx* mice transplanted with HMDSCs**

	6 wk after injection (7[a])	9 wk after injection (13[a])	19 wk after injection (9[a])	Secondary recipients 6 wk after injection (5[a])
	%	%	%	%
CD90	3	5.8	6	2
CD45	22	32	34	12
CD43	~0.1	~0.4	1	0
CD11	5.5	7.5	8	3
Gr-1/Mac1	33.6	45.5	66	11
B220	2.3	1.7	3	1.5
CD4	1.9	1.9	3.4	1.8
CD8	2	2.3	3.6	2.1
Sca-1	~0.1	~0.1	~0.1	~0.1
CD34	~0.2	~0.2	~0.18	~0.1

Blood chimerism of donor-derived (H-2d) cells after transplantation of 50×10^5 Sca-1$^+$/CD34$^-$/L-selectin$^+$ from the muscles of donor Balb/c mice (H-2d) mixed with 1×10^7 bone marrow–derived cells from host C57BL10J *mdx/mdx* mice (H-2b). Repopulation of lymphoid and myeloid cell compartments in secondary recipients injected with bone marrow of rescued lethally irradiated *mdx/mdx* mice. Results are expressed as mean percentage.
[a]Number of total animals treated.

referred to as homing MDSCs (HMDSCs). To increase the percentage of HMDSCs, we cloned the Sca-1$^+$/CD34$^-$ MDSCs obtained from newborn Des-LacZ mice. Among the 30 clones produced, two (named G13 and F9) expressed the L-selectin on all cells and CD44 adhesion markers on 30% of these cells (unpublished data). The morphology of these cells was similar to medium-sized blast cells. In a myogenic differentiation medium, G13 and F9 clones lost the L-selectin expression (Fig. 3 a), indicating a specific correlation between the loss of this adhesion molecule and the lineage commitment of these stem cells. These clones produced cells expressing either myosin heavy chain (MyHC) (markers of late myogenic differentiation) or desmin and m-cadherin (markers of quiescent muscle satellite cells), indicating two characteristics of the myogenic potential of the HMDSCs (Fig. 3, d–g). These data were confirmed by immunoblot analysis. Multinucleated MyHC-positive myotubes were also observed after a 14-d culture of Sca-1$^+$/CD34$^-$/L-selectin$^-$ MDSC clones. These results indicate that L-selectin expression in the Sca-1$^+$/CD34$^-$ MDSC subpopulation does not correlate with the capacity to differentiate into muscle cells. Metalloproteinases are a family of molecules that are critical in cell migration and remodeling of the ECM. We observed that HMDSCs expressed higher levels of MMP-2 and MMP-9 than myoblasts obtained after HMDSC commitment (Fig. 3 h). These data support the notion that the high migratory capacity of HMDSCs is the result of a combination of factors.

Quantification and distribution of attachment to blood vessels of clonally expanded HMDSCs

To quantify the inhibition of the binding of HMDSC clone G13 to the muscle blood vessels produced by the L-selectin antibody, we labeled these cells by growing them for 4 h with 200 μCi/ml [^{3}H]glycerol. This quantification method was optimized to detect a small number of accumulated cells. The cells were divided in two pools, one was treated with an anti–L-selectin antibody at 50 μg/ml. Anti–L-selectin was found to be functional, as assessed by binding/blocking assays with an L-selectin–transfected cell line (unpublished data). Each pool of cells (5×10^5 cells per animal) was injected intravenously in two groups of *mdx*/*mdx* mice. Group 1 was 2 mo old ($n = 5$), and group 2 was 1 yr old ($n = 5$). The radioactivity was compared 2 h after HMDSC injection. To demonstrate that the radioactivity detected in the engrafted tissues was only due to the HMDSC accumulation and not to nonspecific leaking of radioactivity, we injected cell lysates from radiolabeled cells as a control (Table II). We found a low percentage of tissue incorporation of lysate-derived radioactivity (mostly in the liver). Moreover, to exclude the potential effects of the complement, five *mdx*/*mdx* animals were injected with HMDSCs pretreated with the F(ab')$_2$ anti–L-selectin. This treatment revealed similar tissue distribution in comparison to the animals injected with the HMDSCs treated with anti–L-selectin (Table II). After the injection of intact HMDSCs not treated with anti–L-selectin, the radioactivity was detected prevalently in muscle tissues such as TA, quadriceps, pectoral, diaphragm, and triceps brachii muscles but not in lungs, liver, and kidneys of 2-mo-old *mdx*/*mdx* mice. However, a significant decrease in muscle radioactivity ($P < 0.001$) was observed in 2-mo-old

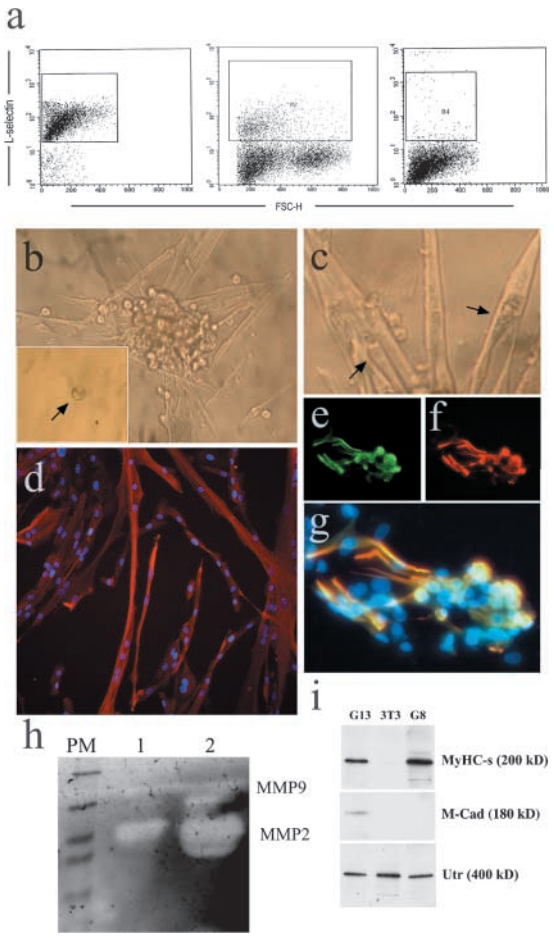

Figure 3. **Myogenic differentiation of Sca-1$^+$/CD34$^-$ MDSCs in cultures.** Characterization of Sca-1$^+$/CD34$^-$ MDSCs from Rosa26 and Des-LacZ transgenic mice for the L-selectin expression (a). L-selectin expression by Sca-1$^+$/CD34$^-$ MDSCs (a, first screen) was decreased when these cells differentiated in myoblasts (a, second screen) and lost when they formed myotubes (a, third screen). For cloning, single cells within the Sca-1$^+$/CD34$^-$ MDSCs (b, inset) were plated in proliferative conditions described in the Materials and methods section. Sca-1$^+$/CD34$^-$/L-selectin$^+$ cells (HMDSCs) obtained from clone G13 differentiated well into myotubes (b and c) expressing the late myogenic marker MyHC (d). Few round single cells near myotubes were positive for the expression of m-cadherin (e) and desmin (f) markers. Panel g corresponds to the merging of e and f. Metalloproteinase activity of the HMDSCs (h, lane 2) was also tested and compared with that of myoblasts (h, lane 1). (i) Immunoblotting analysis of slow MyHC and m-cadherin by HMDSCs obtained from clone G13. The first lane of all immunoblottings corresponds to a homogenate of clone G13. 3T3 fibroblast and G8 myoblast cell lines were used as controls. Utrophin immunoblotting indicated that the same total protein concentrations were present in all specimens.

mdx/*mdx* mice injected with HMDSCs treated with anti–L-selectin (Table II). Muscles of 1-yr-old *mdx*/*mdx* injected animals revealed a marked reduction of the radioactivity ($P < 0.001$) and no difference between animals injected with HMDSCs and HMDSCs treated with anti–L-selectin (Fig. 5; Table II). Organs showed no significant differences in distribution between both groups of *mdx*/*mdx* mice, as shown in Table II. Note also that the level of radioactivity was much lower in organs than in muscles. We also qualitatively assessed the muscle localization of [^{3}H]glycerol HMDSC clone G13

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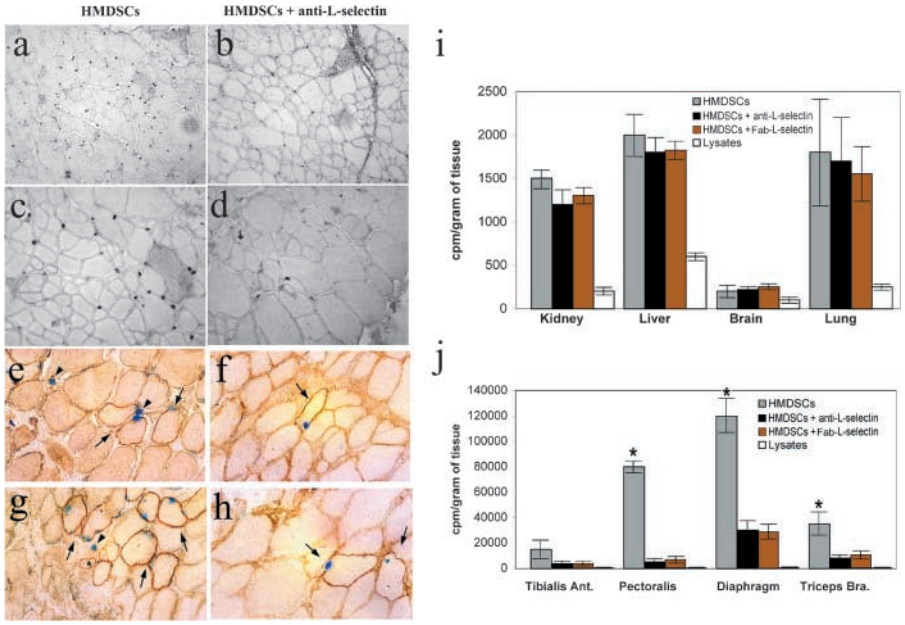

Figure 4. **Localization in the muscle, by autoradiography, 2 h after the i.v. injection of HMDSC clone G13 labeled with [^{3}H]glycerol.** The [^{3}H]glycerol-labeled cells were distributed around the muscle fibers and near the muscle vessels (a–d). More [^{3}H]glycerol-labeled cells were observed per section of the pectoralis muscle in the group of *mdx* mice injected with HMDSCs without the anti–L-selectin treatment (a, 20×; c, 40×) than in the group treated with anti–L-selectin (b, 20×; d, 40×). HMDSCs participated actively after i.v. injection to muscle formation in *mdx* mice. A small number of β-gal–positive nuclei were observed in myofibers 60 d after injection in muscles of HMDSCs obtained from Des-LacZ transgenic mice, thus having a nuclear LacZ reporter gene (e and g). Fewer labeled nuclei were observed in the muscles of mice pretreated with anti–L-selectin (f and h). In these experiments, the myofibers containing Des-LacZ–positive nuclei coexpressed the normal dystrophin protein after immunoperoxidase reaction (e–h, arrows). Some LacZ-positive cells (e, arrowheads) were also found near regenerating myofibers. Radioactivity was significantly increased 2 h after the injection of HMDSCs in muscles of *mdx* mice compared with muscles injected with HMDSCs + anti–L-selectin (*, $P < 0.001$) (j). Organ analysis showed no statistically significant differences in radioactivity between groups receiving HMDSCs versus HMDSCs + anti–L-selectin at the observed time point (i.e., 2 h) (i). To exclude the potential effects of the complement, *mdx*/*mdx* animals were also injected with HMDSCs pretreated with F(ab')$_2$ anti–L-selectin. Lysates from radiolabeled cells were injected as a control for nonspecific binding of radioactivity.

by autoradiography. [^{3}H]glycerol-labeled cells were distributed around the muscle fibers and near the muscle blood vessels. The number of [^{3}H]glycerol-labeled cells per section reflected the level of radioactivity detected in the tissue (Fig. 4, a–d). Donor [^{3}H]glycerol-labeled cells were indeed found in the pectoral, soleus, gastrocnemius, TA, and triceps brachii of

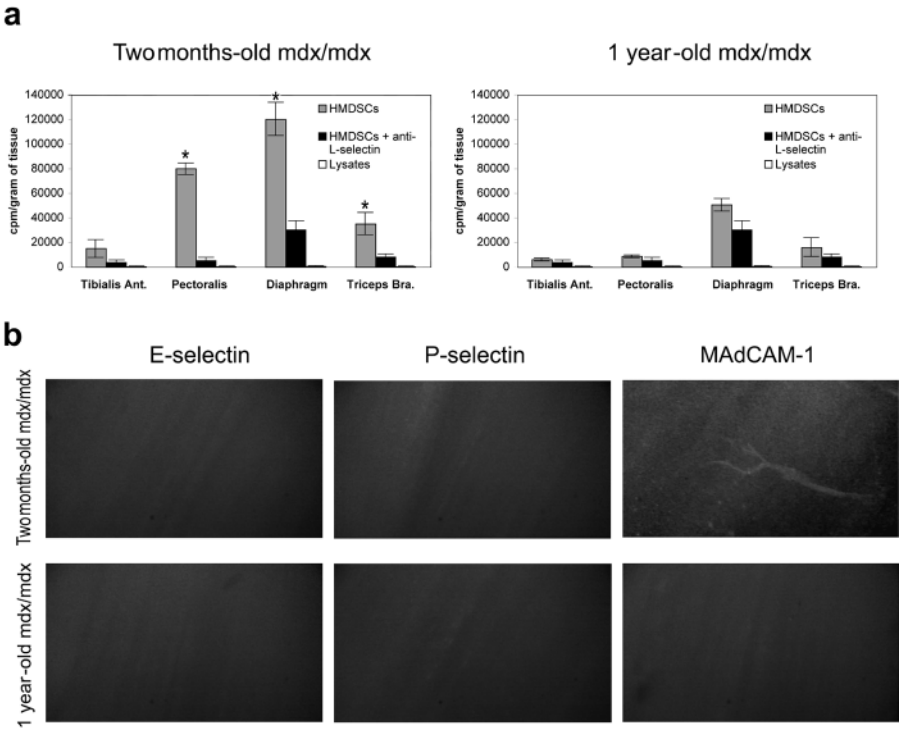

Figure 5. **Muscle migration of HMDSCs in dystrophic muscle is age dependent.** The radioactivity was significantly increased 2 h after the i.v. injection of HMDSC clone G13 labeled with [^{3}H]glycerol in muscles of 2-mo-old *mdx*/*mdx* mice receiving HMDSCs versus 1-yr-old dystrophic animals injected with the HMDSCs ($P < 0.001$). The radioactivity levels in the diaphragm and pectoralis muscles of 2-mo-old *mdx*/*mdx* mice were respectively six- and fivefold higher than in muscles injected with HMDSCs treated with anti–L-selectin (*, $P < 0.001$). Muscle radioactivity levels in older (1-yr-old) *mdx*/*mdx* mice injected with clone G13 are not significantly different from those in *mdx*/*mdx* mice injected with HMDSCs treated with anti–L-selectin. (b) Expression of L-selectin ligands on endothelium from dystrophic muscle by using intravital microscopy. Fluorescently labeled anti–E-selectin, –P-selectin, and –MAdCAM-1 antibodies were injected in *mdx* mice, and positivity was determined on the vascular endothelium of the dystrophic muscles. Dystrophic muscles from 2-mo-old *mdx*/*mdx* mice expressed MAdCAM-1 at venular sites, but not E- and P-selectin, while aging dystrophic vessels did not express L-selectin ligands on their surface.

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Table II. **Tissue distribution of [^{3}H]glycerol-labeled HMDSCs after the injection of *mdx* mice**

	HMDSCs	HMDSCs + anti–L-selectin	HMDSCs + F(ab) anti–L-selectin	Cell lysates
	%/g	%/g	%/g	%/g
Soleus muscle	31 ± 0.05	8.72 ± 0.09	9.02 ± 0.1	1.24 ± 0.01
Pectoralis muscle	22.45 ± 0.10[a]	3.2 ± 0.37	3.3 ± 0.41	0.32 ± 0.04
Tibialis anterior muscle	12.15 ± 0.01	3.5 ± 0.53	3.55 ± 0.65	0.7 ± 0.02
Triceps brachialis muscle	5.31 ± 0.02[a]	1.2 ± 0.01	1.23 ± 0.07	1.18 ± 0.01
Diaphragm	33.05 ± 0.07[a]	6.18 ± 0.02	6.18 ± 0.06	0.12 ± 0.21
Liver	2.34 ± 2.10	1.2 ± 0.07	1.25 ± 0.09	6.23 ± 0.01
Kidney	4.74 ± 0.37	1.8 ± 0.02	1.9 ± 0.04	1.44 ± 0.05
Lung	1.5 ± 0.01	1.3 ± 0.15	1.1 ± 0.25	3.6 ± 0.02
Bone marrow	1.8 ± 0.55	0.9 ± 0.32	0.7 ± 0.56	0.54 ± 0.1
Brain	0.28 ± 0.2	0.2 ± 0.1	0.21 ± 0.15	1.18 ± 0.2

Radioactivity distribution in muscles and visceral organs of 2-mo-old *mdx*/*mdx* mice after the injection of [^{3}H]glycerol-labeled HMDSCs with or without anti–L-selectin or the injection of cell lysates. Results are expressed as percentage of injected activity per gram of tissue (± SD) 2 h after i.v. injection.
[a]$P < 0.01$ vs. HMDSCs + anti–L-selectin.

injected mice. Lungs, liver, brain, and kidneys contained only a few labeled cells (unpublished data), which corresponded with the low radioactivity detected in these tissues (Table II; Fig. 4 i). Tissues from mice not injected with HMDSCs scored negatively (Fig. 4, i and j).

Vascular endothelium from *mdx*/*mdx* muscle expresses MAdCAM-1

To further support our results on HMDSCs, we performed an in vivo study aimed at identifying potential L-selectin ligands on endothelium from dystrophic muscle. To determine whether E- and P-selectin and MAdCAM-1 are expressed on vascular endothelium from *mdx* striate muscle, we injected Alexa®488–labeled mAb anti–E-selectin, –P-selectin, or –MAdCAM-1. 20 min after mAb administration, anti–E-selectin, –P-selectin, or –MAdCAM-1 antibody accumulation was studied with videomicroscopy (Fig. 5). As shown in Fig. 5, no positivity for anti-selectin mAbs was detected in 2-mo-old *mdx* mice. In contrast, positivity for anti–MAdCAM-1 antibody was detected on striate muscle venules of 2-mo-old animals, supporting the data that we obtained with anti–L-selectin blocking antibody. Interestingly, when we studied the expression of vascular adhesion molecules in older *mdx* mice (12 mo old) no positivity was detected for all the Alexa®488–labeled antibodies. However, positivity for the anti–MAdCAM-1 antibody was detected in high endothelial venules from only young *mdx*/*mdx* mice, suggesting that MAdCAM-1 may be selectively down-regulated during aging on the vascular endothelium of dystrophic muscle. An isotype-matched control antibody revealed no positivity in striated muscles from young and old *mdx* mice (unpublished data).

Expression of LacZ and dystrophin in *mdx* mice injected with HMDSCs

We then investigated the in vivo ability of HMDSCs to differentiate into the myogenic lineage after i.v. injection. Tissues, shown by radioactivity studies to contain donor cells, were evaluated for the expression of LacZ and dystrophin transcripts 3 mo after HMDSC clone G13 engraftment. The expression of LacZ and dystrophin was investigated by histochemical staining of cryostat sections of muscles, brain, liver, lungs, kidneys, and bones of the same host mice. β-Gal–positive nuclei were detected only in striated muscles of the *mdx* mice. We observed that ~2% of fibers of a cross section of the pectoralis muscle were not only dystrophin positive but also contained β-gal–positive nuclei (Fig. 4 e). In the other muscles, the percentage of dystrophin-positive muscle fibers with β-gal nuclei was much lower (~0.5–1.0% of total fibers in a given cross section) (Fig. 4 g). The dystrophin antibody did not cross-react with autosomal dystrophin-related proteins, such as utrophin. Control *mdx* muscles contained rare revertant fibers that were dystrophin positive (Hoffman et al., 1990) but β-gal negative.

Discussion

Previous studies have demonstrated the capacity of stem cells to engraft and participate in muscle repair after a systemic delivery, but they have not identified the mechanism responsible for the muscle homing of these stem cells (Goodell et al., 1996, 1997; Ferrari et al., 1998). The factors that induce stem cells to migrate may be tissue specific or generic to injury. In the case where we want to verify whether stem cells migrate after injury, a damage to the muscle with a needle can induce an inflammatory reaction and muscle homing. Intramuscular injection of MDSCs into *mdx*/*mdx* mice resulted in a rapid mobilization and incorporation of donor cells into several distant muscles and bone marrow. As dystrophic *mdx* mice have frequent muscle fiber damage due to the absence of dystrophin (Hoffman et al., 1987), our results in *mdx* mice support the notion that injury provides a sufficient muscle homing.

Using the intravital microscopy method, we identified a subpopulation of Sca-1$^+$/CD34$^-$ MDSCs that are responsible for the migration to the mesodermal derivatives (muscle and bone marrow) after their i.m. transplantation sharing the same properties and phenotype (Sca-1$^+$, CD34$^-$, and lineage marker negative) as the so-called SP. We found that only ~30% of the Sca-1$^+$/CD34$^-$ MDSCs express the L-selectin adhesion molecule. Clones of these stem cells resulted in a corresponding enrichment in the transplantable muscle content of this subset. Moreover, these clones expressed a high level of MMP-2 and MMP-9, two matrix-degrading metalloproteinases that play an important role in

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tissue remodeling and in processes involved in cell migration (Birkedal-Hansen et al., 1993).

Sca-1$^+$/CD34$^-$/L-selectin$^+$ MDSCs (HMDSCs) did not "home" specifically to the muscle tissues. We found, however, a few of them in spleen, liver, lung, and brain after i.v. injection. Thus muscle homing was probably related to a high expression of the L-selectin ligands on the vessels of inflamed muscles, as is the case for dystrophic muscles (Berenson et al., 1988). Importantly, we found that endothelium from dystrophic muscle expresses MAdCAM-1, but not E- and P-selectin. MAdCAM-1 is a ligand for L-selectin that expressed selectively at venular sites of lymphocyte extravasation into mucosal lymphoid tissues and lamina propria, where it directs local lymphocyte trafficking (Berg et al., 1993; Briskin et al., 1993). It has been previously shown that MAdCAM-1 is also expressed on chronically inflamed endothelium from nonlymphoid organs and mediates the recruitment of leukocytes into sites of inflammation (Yang et al., 1997; Connor et al., 1999; Hillan et al., 1999; Souza et al., 1999; Kanwar et al., 2000). Our results represent the first demonstration that vascular endothelium from dystrophic muscle is able to express MAdCAM-1. Interestingly, we show that only blood vessels from young *mdx* mice express MAdCAM-1, whereas older mice down-regulate the mucosal addressin from their surface.

Although antibody against L-selectin reduced muscle homing of HMDSCs, some attachment of HMDSCs to the muscle tissues was still observed after this blockade. The remaining homing capacity could be attributed either to an incomplete blocking of all L-selectin molecules on the HMDSCs or to the rapid expression of new L-selectin sites on these cells. However, an alternative hypothesis is that other cell adhesion molecules may be involved in the in vivo muscle homing of HMDSCs. Collectively, these findings showed that muscle homing may result from a mixture of pathways including tissue-specific interactions between L-selectin adhesion molecules of stem cells and ligands present on the endothelium layer of blood vessels after local damage.

The absence of L-selectin antigen after the commitment of HMDSCs indicates a possible functional role in the maintenance of a "primitive" state of multipotent stem cells that coincides with their homing properties. In this regard, it is interesting that L-selectin is also expressed in the earliest hematopoietic stem/progenitor cells (HSPCs) and in intermediate stages of leukocyte development, and all erythroid and megakaryocytic lineage cells are devoid of L-selectin expression (Kansas et al., 1990; Terstappen et al., 1992; Sackstein, 1997). Moreover, several studies have indicated L-selectin as a "bone marrow homing molecule" (Mazo et al., 1998; Greenberg et al., 2000), suggesting a role for L-selectin adhesive interactions with its ligand in the creation and/or perpetuation of HSPC microenvironmental niches after hematopoietic stem cell transplantation (Dercksen et al., 1995; Watanabe et al., 1998; Koenig et al., 1999). In agreement with these data, we observed a reconstitution of the hematopoietic compartment of lethally irradiated *mdx*/*mdx* recipients after injection of HMDSCs.

The Sca-1$^+$/CD34$^-$/L-selectin$^+$ cells differentiate well in vitro in myogenic lineage and can rescue the dystrophic phenotype after their i.v. injection into *mdx*/*mdx* mice. However, the muscle dystrophin restoration was below the levels needed to provide clinical benefits in DMD and not in correspondence with the high attachment to muscle blood vessels observed 2 h after i.v. injection of HMDSCs. It is possible that these cells are in competition with the resident stem cells for muscle fiber repair. In the case of bone marrow transplantation, the damage is usually whole body irradiation or other myeloablative agents (Hendrikx et al., 1996). Both approaches probably kill bone marrow stem cells and result in a free stem cell niche where the injected bone marrow stem cells can home (Jackson et al., 1999; Wright et al., 2002) and have no competition. The presence of only a few HMDSCs in the bone marrow of *mdx*/*mdx* mice not irradiated and the total reconstitution obtained after irradiation agree with this interpretation. Additionally, muscle incorporation of the HMDSCs initially attached to the muscle blood vessels may prevalently generate satellite cells (as indicated by in vitro differentiation experiments), which did not fuse with existing muscle fibers in the time course of this experiment.

The HMDSCs attached initially to the muscle blood vessels may remain as a multipotent circulating pool of cells (Delassus and Cumano, 1996; Shi et al., 1998) and eventually migrate to other tissues during their life span. The progressive muscle degeneration and vessel wall calcification present in the dystrophic muscles may significantly reduce the migration of HMDSCs in the damaged muscle tissue (unpublished data). In the future, stem cell transplantation could be optimized by performing them during the initial stage of DMD disease to provide levels of muscle engraftment that would be clinically useful. Our results show an important observation that will help to start the investigation of the mechanism of muscle homing, and this may eventually aid in improving the efficacy of the systemic delivery of stem cells to repair dystrophic muscles.

Materials and methods

Transgenic mice

This work was authorized and supervised by the CHUQ Animal Care Committee and was conducted according to the guidelines set by the Canadian Council of Animal Care. Muscle-derived cells were established from the ROSA-26 animals (Jackson ImmunoResearch Laboratories) and the Des-LacZ strain (gift from D. Paulin, Paris University, Paris, France) where the β-gal gene was expressed only in skeletal muscle (Li et al., 1997).

Isolation of MDSCs

Muscle-derived cells were isolated using a previously described protocol (Rando and Blau, 1994; Torrente et al., 2001) and preplated in noncoated flasks with Ham's F10 supplemented with 20 μg/ml pancreatic insulin, 50 ng/ml stem cell factor, 15% FBS, and 1% penicillin/streptomycin. All the culture medium supplies were purchased from GIBCO BRL. Sca-1$^+$ cells were purified by positive selection by using the Sca-1 multisort kit (Miltenyi Biotec). After the isolation of Sca-1$^+$ cells, multisort microbeads were removed using the MACS® multisort release reagent, and the Sca-1$^+$ cells were incubated with a biotinated, conjugated CD34 antibody (1/50) and resuspended with an antibiotin conjugated with paramagnetic microbeads (Miltenyi Biotec). After selection, aliquots of the Sca-1$^+$/CD34$^+$, Sca-1$^+$/CD34$^-$, Sca-1$^-$/CD34$^+$ cell fractions were analyzed to assess purity. For two-color flow cytometry, FITC–anti-CD34 (BD Biosciences) and phycoerythrin-conjugated anti–Sca-1 Mab (Miltenyi Biotec) were used as previously described (Forni, 1979; Gehling et al., 1997; Lange et al., 1999; Nakamura et al., 1999). Flow cytometric analysis was performed on a FACScan® flow cytometer using Cell Quest software (Becton Dickinson) with 10,000 events recorded for each sample.

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Clonogenic and differentiation potential of Sca-1$^+$/CD34$^-$ MDSCs

For clonal analysis, the Sca-1$^+$/CD34$^-$ isolated cells were resuspended in uncoated wells of 96-well plates (1 cell/well) and cultured with the proliferating medium as above. Only the wells containing a single cell were used. The ability of Sca-1$^+$/CD34$^-$ MDSCs to undergo differentiation into myogenic lineage was tested with Ham's F10 supplemented with 5% FBS, and after 14 d of culture, differentiated myoblasts were detected by immunostaining with antibodies against desmin (1:100; Sigma-Aldrich), MyHC (1:200; Ylem), and m-cadherin (1:50; Santa Cruz Biotechnology, Inc.). For Western blot analyses, 100 μg of extracted proteins was separated on 6% polyacrylamide gels and electrotransferred onto nitrocellulose membranes (Bio-Rad Laboratories) and incubated with monoclonal antibodies directed against either dystrophin, utrophin, or m-cadherin and revealed using a commercially available chemiluminescence kit (Ultra ECL; Pierce Chemical Co.). Metalloproteinase activity was assessed using 10% gelatin zymogram gels, as described previously (El Fahime et al., 2000).

Intramuscular transplantation of MDSCs into *mdx*

5×10^5 MDSCs prepared from newborn male F1 Balb/c(H2d)-C57BL10J(H2b) mice and Sca1$^+$/CD34$^-$ MDSCs from newborn male Des-LacZ mice (H2b) were injected into the right TA of three groups each of five female 2-mo-old C57BL/10ScSn *mdx*/*mdx* (H2b) mice. Mice were anesthetized with an i.p. injection of physiologic saline (10 ml/kg) containing ketamine (5 mg/ml) and xylazine (1 mg/ml). Detection of chromosome Y was performed as previously described (Caron et al., 1999). To verify tolerance induction after transplantation, we examined Balb/c skin grafts placed on C57BL/10ScSn *mdx*/*mdx* mice. Skin from the tails of Balb/c donors was engrafted to the backs of three C57BL/10ScSn *mdx*/*mdx* recipients (Rosenberg, 1991). The grafts were examined daily for evidence of edema, hair growth, and rejection.

Intravital microscopy

Muscle homing and vascular adhesion properties were also verified into the pectoral muscle of three groups of *mdx*/*mdx* mice ($n = 15$) injected into the TA muscles with 5×10^5 of each subpopulation of MDSCs (i.e., Sca-1$^-$/CD34$^+$, Sca-1$^+$/CD34$^+$, and Sca-1$^+$/CD34$^-$) as previously described (Torrente et al., 2001). An intravital microscope (Olympus BX50WI) equipped with water immersion objectives (Olympus Achroplan; focal distance 3.3 mm, NA 0.5) was used for these experiments. The MDSCs were fluorescently labeled with 2′,7′-bis-(carboxyethyl)-5(and-6) carboxy-fluorescein (BCECF; Molecular Probes). All scenes were recorded on videotape using a silicon-intensified target video camera (VE 1000-SIT; Dage MTI), a time and frame counter (ELCA), and a high picture quality SVHS Panasonic VCR.

Intravenous transplantation of MDSCs into lethally irradiated *mdx* mice

The Sca-1$^+$/CD34$^-$ MDSCs and bone marrow cells were obtained from Balb/c newborn mice (H-2^d haplotype) and injected intravenously into lethally irradiated (950 cGy) C57BL/10ScSn *mdx*/*mdx* (H-2^b haplotype). Animals were then followed for 8 wk. No changes in general health status were noted in recipient mice. 20 C57BL/10ScSn *mdx*/*mdx* mice received 5×10^7 bone marrow–derived cells. 20 C57BL/10ScSn *mdx*/*mdx* mice received both the Sca-1$^+$/CD34$^-$ MDSCs (50×10^4) and bone marrow–derived cells (5×10^6). Two groups served as controls. Five C57BL/10ScSn *mdx*/*mdx* mice received pooled bone marrow cells from Balb/c mice after irradiation (control 1). Four C57BL/10ScSn *mdx*/*mdx* mice were irradiated and did not receive cell transplantation to determine whether the irradiation dose chosen was lethal (control 2). All four of these mice died within 2 wk. For transplantation into secondary recipients, bone marrow was harvested from a rescued *mdx*/*mdx* mouse, and 8×10^5 mononucleated cells were injected into each of five *mdx*/*mdx* recipients, prepared as described above.

In vivo histochemistry and immunocytochemistry

For histochemistry on tissue sections, samples were frozen in liquid nitrogen–cooled isopentane and cryostat sectioned. Slides were examined by light microscopy for β-gal–positive myofibers (Li et al., 1993). Detection of dystrophin was performed with a polyclonal antibody against the COOH 60-kD Dys fragment (gift of J.S. Chamberlain, University of Michigan, Ann Arbor, MI). Antibodies were visualized using an HRP-coupled secondary antibody (Bio-Rad Laboratories). The Lac-Z, dystrophin double-positive myofibers were counterstained with hematoxylin and eosin. A fluorescence microscope (Leica DMIR2) equipped with Leica Qfluoro software was used for these experiments.

Characterization of muscle homing by [^{3}H]glycerol labeling and autoradiography

5×10^5 HMDSCs were incubated overnight at 37°C in RPMI supplemented with 10 μCi/ml [^{3}H]glycerol (Dupont), as previously described (Constantin et al., 1997), and injected intravenously into *mdx*/*mdx* mice. Animals were killed 2 h later, muscle, lung, kidney, liver, and blood tissues were collected, and radioactive content was measured in a β-counter (LS1801; Beckman Coulter). We compared the distribution of radioactivity of five 2-mo-old *mdx*/*mdx* mice injected i.v. with 10×10^5 HMDSCs with five dystrophic animals injected with HMDSCs pretreated for 15 min at room temperature with 100 μg/μl anti–L-selectin MEL-14 (gift from E.C. Butcher, Stanford University, Stanford, CA). To assure a longer blocking of the L-selectin on HMDSCs, L-selectin pretreatment was accompanied by i.v. injection of anti–L-selectin (400 μg/ml of antibody diluted in 500 μl of sterile HBSS). To exclude the potential effects of the complement, the F(ab′)$_2$ fragment of anti–L-selectin was generated using the immunoPure F(ab′)$_2$ preparation kit (Pierce Chemical Co.) as previously described (Lamoyi, 1986). In this experiment, five *mdx*/*mdx* animals were injected with HMDSCs pretreated for 15 min at room temperature with 100 μg/μl of F(ab′)$_2$ anti–L-selectin. For histologic localization of radiolabeled cells, 8-μm frozen sections of muscle tissues were placed on Histostick-coated slides (Accurate Chemicals), fixed with methanol, and washed with PBS. The slides then were dipped in Kodak NTB 2 emulsion (Eastman Kodak Co.) and exposed for 6 wk at 4°C. The slides were developed and fixed using EDF/EDP Photochemicals (Eastman Kodak Co.) according to the processing protocol from the manufacturer.

In vivo staining of endothelial adhesion molecules

MAbs anti–E-selectin, –P-selectin, and –MAdCAM-1 and an isotype-matched control antibody (anti–human Ras) were labeled using the Alexa Fluor®488 labeling kit (Molecular Probes). 50 μg fluorescent mAb was injected intravenously. 20 min later, the animal was anesthetized and perfused through the left ventricle with cold PBS (Piccio et al., 2002). Striate muscle vessels were visualized using the intravital microscopy setting as described for intravital microscopy experiments. Anti–L-selectin mAb Mel-14 was from American Type Culture Collection. Anti–E-selectin and –P-selectin antibodies (RME-1 and RMP-1) were obtained as previously described (Walter et al., 1997a,b). Anti–MAdCAM-1 was provided by E.C. Butcher. Green CMFDA (5-chloromethylfluorescein diacetate) for the labeling of HMDSCs for intravital microscopy was prepared as a stock solution in DMSO and kept at −20°C until the moment of use.

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