Table 1. Cells that express β-gal in Gnls Tg and DUE Tg mice and comparison with endogenous utrophin expression

Tissue	Endogenous utrophin	β-Gal expression		
		Gnls	DUE line 1	DUE line2
Liver	Surface of hepatocyte	Hepatocyte	Hepatocyte	Hepatocyte
Testis	BM of seminiferous tubule	Sertoli cell	Sertoli cell	Sertoli cell
	Leydig cell	Leydig cell	Leydig cell	Leydig cell
Colon	BM of large intestinal gland	Goblet cell	Goblet cell	Goblet cell
	Muscularis mucosa	ND	ND	ND
Submandibular gland	BM of serous & mucous acinus	Serous & mucous secretory cell	Serous & mucous secretory cell	Serous & mucous secretory cell
Small intestine	BM of villus & crypt	Paneth cell, goblet cell	Paneth cell, Goblet cell	Paneth cell, Goblet cell
	Muscularis mucosa	ND	ND	ND
Kidney	BM of cortical renal tubule	Epithelial cell of cortical renal tubule	Epithelial cell of cortical renal tubule	Epithelial cell of cortical renal tubule
	BM of collecting duct in renal medulla			
	Glomerulus	ND	ND	ND
Lung	Alveolus	Alveolar cell	Alveolar cell	Alveolar cell
	Terminal bronchiole epithelium	ND	ND	ND
Cerebrum	Choroid plexus	ND	Ependymal cell of choroid plexus	ND
	Pia mater		Fibroblastic cell of pia mater	
Cerebellum	Pia mater	ND	Stellate cell and basket cell of molecular layer	ND
Heart	Intercalated disk	ND	Peripheral cell of intercalated disk	ND
	T tubule			
Skeletal muscle	Neuromuscular junction	ND	Peripheral cell of neuromuscular junction	ND
	Myotendinous junction			
	Regenerating muscle fiber			

The localization of endogenous utrophin is based on this study and previous studies [11,24]. BM, basement membrane; ND, not detected.

Table 2. Summary of β-gal expression in Gnls Tg mice and DUE Tg mice

	Gnls	DUE line 1	DUE line 2
Liver	++	++	±
Testis	+++	+++	+
Colon	++	++	+
Submandibular gland	+++	+++	+
Small intestine	+	+	±
Kidney	±	+	+
Lung	+	++	±
Cerebrum	−	++	−
Cerebellum	−	++	−
Heart	−	++	−
TA muscle	−	+	−

Tg mice were sacrificed at 4–7 weeks, and β-gal expression was examined in several tissues. No β-gal positive nuclei were found in nontransgenic littermates. β-gal expression levels: −, none; ±, trace; +, weak; ++, moderate; +++, strong.

up-regulated. Another study [10] also reported that utrophin transcription was controlled by DUE activity in regenerating muscle and that its activity was dependent on an AP-1 binding site. Injection of marcain into TA muscles of CD1 mice demonstrated elevation of members of the AP-1 factor, *c-fos, fosB, fra-1, fra-2, c-jun, junB* and *junD* [10]; however, we also found distinct elevation of *c-fos* and *fra-1* mRNA in our regeneration system (unpublished observations).

We cultured primary myogenic cells from DUE Tg mice and found that transgene expression was up-regulated during the differentiation process. Moreover, these transgene expression patterns corresponded to the endogenous utrophin expression profile. This result indicates that the participation of DUE in utrophin expression during muscle regeneration might depend largely on DUE activity in the later stage of muscle differentiation. It is intriguing to note that transgene and endogenous utrophin expression patterns coincided with the expression profile of MEF2C, but not that of myogenin. It has been already reported that MEF2C mediates the promoter activity of c-jun [33]. The MEF2C-c-jun pathway is one of the candidates for regulation of utrophin expression via DUE. Analysis of the transcriptional factors that interact with DUE sequences, particularly the AP-1 site, would be very intriguing and should be clarified by a future study.

In the present study, we also demonstrated that the addition of DUE augmented transgene expression not only in the heart and skeletal muscle, but also in other tissues, such as the cerebral pia mater and choroid plexus and the cerebellar choroid plexus and molecular layer. In addition, transgene expression was elevated in the kidney and lung of DUE Tg mice compared to that of Gnls Tg mice, although it is necessary to consider the difference in transgene copy numbers. These results suggest that DUE activity is not muscle specific, consistent with the data of Galvagni *et al.* [26]. In their study, a construct of DUE added to the utrophin promoter was transiently transfected to various cells and revealed that DUE enhanced utrophin promoter activity not only in C2C12 myoblasts, but also in HeLa cells and RD cells.

However, the addition of DUE cannot fully explain the transcriptional regulation of utrophin. In the cerebrum and cerebellum, endogenous utrophin was expressed in the pia mater and choroid plexus. We found β-gal-positive

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nuclei in the cerebral pia mater along the basal lamina, but did not find many β-gal-positive nuclei in the cerebellum. There are several possibilities to explain this discrepancy. The first possibility is that the domain that regulates utrophin expression in the pia mater of the cerebellum is different from that for the pia mater in the cerebrum. The second possibility is that transcription of utrophin might be less active in fibroblastic cells of the pia mater of cerebellum compared to those in the cerebrum. However, a fundamental difference between fibroblastic cells in the cerebrum and those in the cerebellum has not been reported; further experiments are required to explain this discrepancy.

We demonstrated that DUE is necessary for utrophin expression in skeletal muscle, but the increase in the utrophin expression level was much larger than the transgene expression in regenerated muscle. Another study [11] also detected the increase in the abundance of A-utrophin protein in muscle from *mdx* mice but could not find any parallel elevation in the levels of utrophin transcripts. Therefore, A-utrophin expression may also be regulated at the post-transcriptional level. Indeed, recent studies have shown that distinct cis-acting elements within the utrophin 3′-UTR were important not only for controlling the stability of utrophin transcripts in muscle cells, but also for targeting them to specific subcellular locations [34,35].

Post-translational levels are also important for utrophin expression through stabilization of the protein. DAPs such as dystrophin, β-dystroglycan, α-dystroglycan, and α-sarcoglycan have been linked to regulation by protein degradation mechanisms including the ubiquitin-proteasome pathway [36] and calpain-mediated proteolysis [37]. Inhibition of the proteasomal degradation pathway was found to rescue the expression levels of several DAPs in *mdx* mice [36]. Treatment of normal and DMD human myotubes with glucocorticoid induced utrophin protein without elevations in transcripts, and this was suggested to involve calpain inhibition [38].

It is likely that extrasynaptic expression of utrophin in skeletal muscle of DMD patients would ameliorate the dystrophic pathology, at least to some extent [17,18]. The results of the present study demonstrate that DUE is indispensable to utrophin expression in skeletal muscle and heart. To further investigate the up-regulation mechanisms of utrophin in both tissues, we need to search for transcription factors bound to DUE. In addition, we established primary myogenic cell cultures from DUE Tg mice and found that utrophin up-regulation depends on the DUE motif during muscle differentiation. These cells provide a high through-put screening system for drugs that can up-regulate utrophin expression in myogenic cells.

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Musculoskeletal Pathology

Muscle CD31(−) CD45(−) Side Population Cells Promote Muscle Regeneration by Stimulating Proliferation and Migration of Myoblasts

Norio Motohashi,*† Akiyoshi Uezumi,* Erica Yada,* So-ichiro Fukada,* Kazuhiro Fukushima,*¶ Kazuhiko Imaizumi,† Yuko Miyagoe-Suzuki,* and Shin'ichi Takeda*

From the Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo; the Division for Therapies against Intractable Diseases,‡ Institute for Comprehensive Medical Science, Fujita Health University, Aichi; the Department of Immunology,† Graduate School of Pharmaceutical Sciences, Osaka University, Osaka; the Laboratory of Physiological Sciences,‡ Faculty of Human Sciences, Waseda University, Saitama; and the Third Department of Medicine, Neurology, and Rheumatology,¶ Shinshu University School of Medicine, Matsumoto, Japan*

CD31(−) CD45(−) side population (SP) cells are a minor SP subfraction that have mesenchymal stem cell-like properties in uninjured skeletal muscle but that can expand on muscle injury. To clarify the role of these SP cells in muscle regeneration, we injected green fluorescent protein (GFP)-positive myoblasts with or without CD31(−) CD45(−) SP cells into the tibialis anterior muscles of immunodeficient *NOD/scid* mice or dystrophin-deficient *mdx* mice. More GFP-positive fibers were formed after co-transplantation than after transplantation of GFP-positive myoblasts alone in both *mdx* and *NOD/scid* muscles. Moreover, grafted myoblasts were more widely distributed after co-transplantation than after transplantation of myoblasts alone. Immunohistochemistry with anti-phosphorylated histone H3 antibody revealed that CD31(−) CD45(−) SP cells stimulated cell division of co-grafted myoblasts. Genome-wide gene expression analyses showed that these SP cells specifically express a variety of extracellular matrix proteins, membrane proteins, and cytokines. We also found that they express high levels of matrix metalloproteinase-2 mRNA and gelatinase activity. Furthermore, matrix metalloproteinase-2 derived from CD31(−) CD45(−) SP cells promoted migration of myoblasts *in vivo*. Our results suggest that CD31(−) CD45(−) SP cells support muscle regeneration by promoting proliferation and migration of myoblasts. Future studies to further define the molecular and cellular mechanisms of muscle regeneration will aid in the development of cell therapies for muscular dystrophy. ***(Am J Pathol 2008, 173:781–791; DOI: 10.2353/ajpath.2008.070902)***

Regeneration of skeletal muscle is a complex but well-organized process involving activation, proliferation, and differentiation of myogenic precursor cells, infiltration of macrophages to remove necrotic tissues, and remodeling of the extracellular matrix.[1–3] Muscle satellite cells are myogenic precursor cells that are located between the basal lamina and the sarcolemma of myofibers in a quiescent state, and are primarily responsible for muscle fiber regeneration in adult muscle.[4] Recent studies also demonstrated that a fraction of satellite cells self-renew and behave as muscle stem cells *in vivo*.[5,6] On the other hand, several research groups reported multipotent stem cells derived from skeletal muscle. These include muscle-derived stem cells,[7] multipotent adult precursor cells,[8] myogenic-endothelial progenitors,[9] CD34(+) Sca-1(+) cells,[10] CD45(+) Sca-1 (+) cells,[11] mesoangioblasts,[12] and pericytes,[13] and all were demonstrated to contribute to muscle regeneration as myogenic progenitor cells.

Side population (SP) cells are defined as the cell fraction that efficiently effluxes Hoechst 33342 dye and therefore shows a unique pattern on fluorescence-activated cell sorting (FACS) analysis.[14] Muscle SP cells are proposed to be multipotent[15,16] and are clearly distinguished from satellite

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Address reprint requests to Yuko Miyagoe-Suzuki, M.D., Ph.D., Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187-8502, Japan. E-mail: miyagoe@ncnp.go.jp.

cells.[17] Previous reports showed that muscle SP cells participated in regeneration of dystrophic myofibers after systemic delivery[15] and gave rise to muscle satellite cells after intramuscular injection into cardiotoxin (CTX)-treated muscle.[17] Muscle SP cells adapted to myogenic characteristics after co-culture with proliferating satellite cells/myoblasts *in vitro*,[17] and expressed a satellite cell-specific transcription factor, Pax7, after intra-arterial transplantation.[18] However, the extent to which muscle SP cells participate in muscle fiber regeneration as myogenic progenitor cells is still primarily unknown. Importantly, Frank and colleagues[19] recently showed that muscle SP cells secret BMP4 and regulate proliferation of BMP receptor1α (+) Myf5^{high} myogenic cells in human fetal skeletal muscle, raising the possibility that SP cells in adult muscle play regulatory roles during muscle regeneration.

Previously we showed that skeletal muscle-derived SP cell fraction are heterogeneous and contain at least three subpopulations: CD31(+) CD45(−) SP cells, CD31(−) CD45(+) SP cells, and CD31(−) CD45(−) SP cells.[20] These three SP subpopulations have distinct origins, gene expression profiles, and differentiation potentials.[20] CD31(+) CD45(−) SP cells account for more than 90% of all SP cells in normal skeletal muscle, take up Ac-LDL, and are associated with the vascular endothelium. CD31(+) CD45(−) SP cells did not proliferate after CTX-induced muscle injury. Bone marrow transplantation experiments demonstrated that CD31(−) CD45(+) SP cells are recruited from bone marrow into injured muscle. A few of them are thought to participate in fiber formation.[21] Cells of the third SP subfraction, CD31(−) CD45(−), constitute only 5 to 6% of all SP cells in adult normal skeletal muscle, but they actively expand in the early stages of muscle regeneration and return to normal levels when muscle regeneration is completed. Although CD31(−) CD45(−) SP cells are the only SP subset that exhibited the capacity to differentiate into myogenic, adipogenic, and osteogenic cells *in vitro*,[20] their myogenic potential *in vivo* is limited compared with satellite cells. Therefore, we hypothesized that CD31(−) CD45(−) SP cells might play critical roles during muscle regeneration other than as myogenic stem cells.

In the present study, we demonstrate that the efficacy of myoblast transfer is markedly improved by co-transplantation of CD31(−) CD45(−) SP cells in both regenerating immunodeficient *NOD/scid* and dystrophin-deficient *mdx* mice. We also show that CD31(−) CD45(−) SP cells increased the proliferation and migration of grafted myoblasts *in vivo* and *in vitro*. We further show that CD31(−) CD45(−) SP cell-derived matrix metalloproteinase (MMP)-2 greatly promotes the migration of myoblasts *in vivo*. Our findings would provide us insights into the molecular and cellular mechanisms of muscle regeneration, and also help us develop cell therapy for muscular dystrophy.

Materials and Methods

Animals

All experimental procedures were approved by the Experimental Animal Care and Use Committee at the National Institute of Neuroscience. Eight- to twelve-week-old C57BL/6 mice and *NOD/scid* mice were purchased from Nihon CLEA (Tokyo, Japan). MMP-2-null mice were obtained from Riken BioResource Center (Tsukuba, Japan).[22] GFP-transgenic mice (GFP-Tg) were kindly provided by Dr. M. Okabe (Osaka University, Osaka, Japan). C57BL/6-background *mdx* mice were generously given by Dr. T. Sasaoka (National Institute for Basic Biology, Aichi, Japan) and maintained in our animal facility.

Isolation of Muscle SP Cells

To evoke muscle regeneration, CTX (10 μmol/L in saline; Sigma, St. Louis, MO) was injected into the tibialis anterior (TA) (50 μl), gastrocnemius (150 μl), and quadriceps femoris muscles (100 μl) of 8- to 12-week-old GFP-Tg mice, C57BL/6 mice, MMP-2-null mice, and their wild-type littermates; 3 days later, SP cells were isolated from the muscles as described by Uezumi and colleagues.[20] In brief, limb muscles were digested with 0.2% type II collagenase (Worthington Biochemical, Lakewood, NJ) for 90 minutes at 37°C. After elimination of erythrocytes by treatment with 0.8% NH_4Cl in Tris-buffer (pH 7.15), mononucleated cells were suspended at 10^6 cells per ml in Dulbecco's modified Eagle's medium (Wako, Richmond, VA) containing 2% fetal bovine serum (JRH Biosciences, Inc., Kansas City, KS), 10 mmol/L Hepes, and 5 μg/ml Hoechst 33342 (Sigma), incubated for 90 minutes at 37°C in the presence or the absence of 50 μmol/L Verapamil (Sigma), and then incubated with phycoerythrin (PE)-conjugated anti-CD31 antibody (1:200, clone 390; Southern Biotechnology, Birmingham, AL) and PE-conjugated anti-CD45 (1:200, clone 30-F11; BD Pharmingen, Franklin Lakes, NJ) for 30 minutes on ice. Dead cells were eliminated by propidium iodide staining. Analysis and cell sorting were performed on an FACS VantageSE flow cytometer (BD Bioscience, Franklin Lakes, NJ). APC-conjugated anti-CD90, Sca-1, CD34, CD49b, CD14, CD124, c-kit, CD14 (BD Pharmingen), CD44 (Southern Biotechnology Associates), and CD133 (eBioscience, San Diego, CA) were used at 1:200 dilution.

Preparation of Satellite Cell-Derived Myoblasts and Macrophages

Satellite cells were isolated from GFP-Tg mice or C57BL/6 mice by using SM/C-2.6 monoclonal antibody[23] and expanded *in vitro* in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum and 2.5 ng/ml of basic fibroblast growth factor (Invitrogen, Carlsbad, CA) for 4 days before transplantation. Macrophages were isolated from C57BL/6 mice 3 days after CTX injection. Mononucleated cells were stained with anti-Mac-1-PE (1:200, clone M1/70; BD PharMingen) and anti-F4/80-APC (1:200, clone CI, A3-1; Serotec, Oxford, UK). Mac-1(+) F4/80(+) cells were isolated by cell sorting as macrophages.

Cell Transplantation

To induce muscle regeneration, 100 μl of 10 μmol/L CTX was injected into the TA muscle of *NOD/scid* muscles,

and 24 hours later, 30 μl of cell suspensions containing 3×10^4 myoblasts, 3×10^4 CD31(−) CD45(−) SP cells, or 3×10^4 GFP(+) myoblasts plus 2×10^4 CD31(−) CD45(−) SP cells were directly injected into the TA muscles of 8-week-old *NOD/scid* or *mdx* mice. At several time points after transplantation, the muscles were dissected, fixed in 4% paraformaldehyde for 30 minutes, immersed in 10% sucrose/phosphate-buffered saline (PBS) and then in 20% sucrose/PBS, and frozen in isopentane cooled with liquid nitrogen.

Retrovirus Transduction in Vitro

Red fluorescent protein (DsRed) cDNA (BD Biosciences, San Diego, CA) was cloned into a retrovirus plasmid, pMXs, kindly provided by Dr. T. Kitamura of the University of Tokyo, Tokyo, Japan.[24] Viral particles were prepared by introducing the resultant pMXs-DsRed into PLAT-E retrovirus packaging cells,[25] and the filtered supernatant was added to the myoblast culture. The next day, DsRed(+) myoblasts were collected by flow cytometry.

Immunohistochemistry

We cut the entire TA muscle tissues on a cryostat into 6-μm cross sections, and observed all serial sections under fluorescence microscopy. We then selected two or three sections in which GFP(+) cells were found most frequently. The sections were then blocked with 5% goat serum (Cedarlane, Hornby, Canada) in PBS for 15 minutes, and then reacted with anti-GFP antibody (Chemicon International, Temecula, CA), anti-laminin α2 antibody (4H8-2; Alexis, San Diego, CA), anti-phospho-histone H3 antibody (Upstate Biotechnology, Lake Placid, NY), or anti-DsRed antibody (Clontech, Palo Alto, CA) at 4°C overnight. Dystrophin was detected using a monoclonal antibody, Dys-2 (Novocastra, Newcastle on Tyne, UK), and a M.O.M. Kit (Vector Laboratories, Burlingame, CA). The sections were then incubated with appropriate combinations of Alexa 488-, 568-, or 594-labeled secondary antibodies (Molecular Probes, Eugene, OR) and TOTO-3 (Molecular Probes), and photographed using a confocal laser-scanning microscope system TCSSP (Leica, Heidelberg, Germany). The area occupied by GFP(+) cells or myofibers was measured by using Image J software (National Institutes of Health, Bethesda, MD) on cross sections from three independent experiments, and defined as the distribution area.

RNA Isolation and Real-Time Polymerase Chain Reaction (PCR)

Total RNA was isolated from muscles using TRIzol (Invitrogen). First strand cDNA was synthesized using a QuantiTect reverse transcription kit (Qiagen, Hilden, Germany). The levels of GFP mRNA and 18S rRNA were quantified using SYBR Premix Ex Taq (Takara, Otsu, Shiga, Japan) on a MyiQ single-color system (Bio-Rad Laboratories, Richmond, CA) following the manufacturer's instructions. Primer sequences for real-time PCR were: 18s rRNA, forward: 5′-TACCCTGGCGGTGGGATTAAC-3′, reverse: 5′-CGAGAGAAGACCACGCCAAC-3′ and EGFP, forward: 5′-GACGTAAACGGCCACAAGTT-3′, reverse: 5′-AAGTCGTGCTGCTTCATGTG-3′. The expression levels of *MMP-2* and *MMP-9* were evaluated by conventional reverse transcriptase (RT)-PCR using the following primers: MMP-2, forward: 5′-TGCAAGGCAGTGGTCATAGCT-3′, reverse: 5′-AGCCAGTCGGATTTGATGCT-3′.

Cell Proliferation Assay

CD31(−) CD45(−) SP cells or 10T1/2 cells were cultured in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum for 5 days, and the supernatants were collected as conditioned medium. Myoblasts were plated on 96-well culture plates at a density of 5000 cells/well and cultured in conditioned medium for 3 days. BrdU was then added to the culture medium (final concentration, 10 μmol/L). Twenty-four hours later, BrdU uptake was quantified by a cell proliferation enzyme-linked immunosorbent assay, a BrdU kit (Roche Diagnostics, Meylan, France), and Lumi-Image F1 (Roche).

Gene Expression Profiling

Total RNAs were extracted from CD31(−) CD45(−) SP cells, macrophages, or myoblasts using an RNeasy RNA isolation kit (Qiagen). cDNA synthesis, biotin-labeled target synthesis, MOE430A GeneChip (Affymetrix, Santa Clara, CA) array hybridization, staining, and scanning were performed according to standard protocols supplied by Affymetrix. The quality of the data presented in this study was controlled by using the Microarray Suite MAS 5.0 (Affymetrix). The MAS-generated raw data were uploaded to GeneSpring software version 7.0 (Silicon Genetics, Redwood City, CA). The software calculates signal intensities, and each signal was normalized to a median of its values in all samples or the 50th percentile of all signals in a specific hybridization experiment. Fold ratios were obtained by comparing normalized data of CD31(−) CD45(−) SP cells and macrophages or myoblasts.

In Situ *Zymography*

CD31(−) CD45(−) SP cells, myoblasts, and macrophages were isolated from regenerating muscles 3 days after CTX injection by cell sorting and collected by a Cytospin3 centrifuge (ThermoShandon, Cheshire, UK) on DQ-gelatin-coated slides (Molecular Probes). The slides were then incubated for 24 hours at 37°C in the presence or absence of GM6001 (a broad-spectrum inhibitor of MMPs, 50 μmol/L; Calbiochem, San Diego, CA) or E-64 (a cysteine protease inhibitor, 50 mmol/L; Calbiochem). Fluorescence of fluorescein isothiocyanate was detected with excitation at 460 to 500 nm and emission at 512 to 542 nm.

Statistics

Statistical differences were determined by Student's unpaired *t*-test. For comparison of more than two groups,

one-way analysis of variance was used. All values are expressed as means ± SE. A probability of less than 5% ($P < 0.05$) or 1% ($P < 0.01$) was considered statistically significant.

Results

Marker Expression on Muscle-Derived CD31(−) CD45(−) SP Cells

When incubated with 5 μg/ml of Hoechst 33342 dye at 37°C for 90 minutes, 1 to 3% of muscle mononuclear cells show the SP phenotype (Figure 1A). Previously, we reported that muscle SP cells can be further divided into three subpopulation, CD31(−) CD45(−) cells, CD31(−) CD45(+) cells, and CD31(+) CD45(−) SP cells (Figure 1B).[20] The CD31(−) CD45(−) SP cells did not express Pax3, Pax7, or Myf5, indicating that they are not yet committed to the muscle lineage.[20] RT-PCR suggested that CD31(−) CD45(−) SP cells have mesenchymal cell characteristics.[20] To further clarify the properties of CD31(−) CD45(−) SP cells, we analyzed their cell surface markers. CD31(−) CD45(−) SP cells were negative for CD124, CD133, CD14, c-kit (Figure 1B), and CD184 (data not shown), weakly positive for CD34 and CD49b, and strongly positive for Sca-1, CD44, and CD90 (Figure 1). The FACS patterns shown in Figure 1B suggested that CD31(−) CD45(−) SP cells are a homogeneous cell population. CD14 is an exception. A small fraction of CD31(−) CD45(−) SP cells were strongly positive for CD14, but the majority weakly expressed this marker. The function of CD14^{high} CD31(−) CD45(−) SP cells remains to be determined.

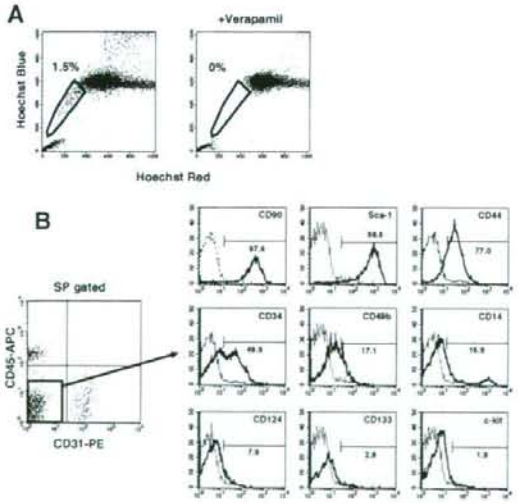

Figure 1. Cell surface markers on CD31(−) CD45(−) SP cells from regenerating muscle. **A:** Mononuclear cells were prepared from limb muscles of C57BL/6 mice at 3 days after CTX injection, incubated with 5 μmol/L Hoechst 33342 with (**right**) or without (**left**) Verapamil, and analyzed by a cell sorter. SP cells are shown by polygons. The numbers indicate the percentage of SP cells in all mononuclear cells. **B: Left:** Expression of CD45 and CD31 on muscle SP cells. **Right:** The expression of surface markers (CD90, Sca-1, CD44, CD34, CD49b, CD14, CD124, CD133, and c-kit) on CD31(−) CD45(−) SP cells was further analyzed by FACS. The *x* axis shows the fluorescence intensity, and the *y* axis indicates cell numbers. Solid lines are with antibodies; dotted lines are negative controls.

Efficiency of Myoblast Transplantation Is Increased by Co-Transplantation of Muscle CD31(−) CD45(−) SP Cells in NOD/scid Mice

To clarify the functions of CD31(−) CD45(−) SP cells during muscle regeneration, we isolated myoblasts from GFP-transgenic mice (GFP-Tg) and injected them (3×10^4 cells/muscle) with or without CD31(−) CD45(−) SP cells (2×10^4 cells/muscle) into TA muscles of immunodeficient *NOD/scid* mice (Figure 2A). CTX was injected into recipient muscles 24 hours before cell transplantation to induce muscle regeneration. Two weeks after transplantation, the contribution of grafted myoblasts to muscle regeneration was investigated by immunodetection of GFP(+) myofibers. Co-transplantation of GFP(+) myoblasts with nonlabeled CD31(−) CD45(−) SP cells produced a higher number of GFP(+) myofibers than transplantation of GFP(+) myoblasts alone (Figure 2, B and C). Furthermore, the average diameter of GFP(+) myofibers was significantly larger in co-transplanted muscles than in muscles transplanted with myoblasts alone (Figure 2D). These results suggest that more myoblasts participated in myofiber formation after co-transplantation than after single transplantation, injected SP cells promoted growth of regenerating myofibers, or both.

Co-transplantation of Myoblasts with Muscle CD31(−) CD45(−) SP Cells Significantly Increased Efficiency of Myoblast Transplantation in mdx Mice

Next, co-transplantation experiments were performed using 8-week-old dystrophin-deficient *mdx* mice as a host. Three kinds of transplantations were performed: 3×10^4 myoblasts derived from GFP-Tg mice, 3×10^4 CD31(−) CD45(−) SP cells derived from GFP-Tg mice, or a mixture of GFP(+) 3×10^4 myoblasts and 2×10^4 CD31(−) CD45(−) SP cells derived from C57BL/6 mice (Figure 3A).

When analyzed at 2 weeks after transplantation, a much higher number of GFP(+) myofibers were detected on cross-sections after co-transplantation of myoblasts and CD31(−) CD45(−) SP cells than after transplantation of GFP(+) myoblasts alone (Figure 3, B and C). On the other hand, transplantation of GFP(+) SP cells alone resulted in formation of few GFP(+) myofibers. This observation is consistent with our previous report.[20] Co-transplantation of myoblasts and CD31(−) CD45(−) SP cells also gave rise to more myofibers expressing dystrophin at the sarcolemma in dystrophin-deficient *mdx* muscles than transplantation of myoblasts alone (data not shown). Again, the diameter of GFP(+) myofibers was significantly larger in co-transplanted muscles than in muscles transplanted with myoblasts or CD31(−) CD45(−) SP cells alone (Figure 3D).

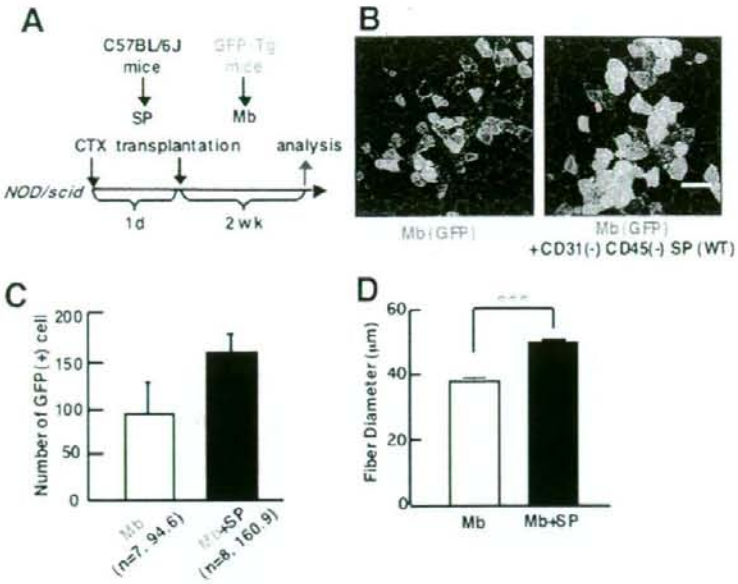

Figure 2. Co-transplantation of myoblasts and CD31(−) CD45(−) SP cells into skeletal muscle of immunodeficient *NOD/scid* mice promotes myofiber formation by transplanted myoblasts. **A:** Schematic protocol of co-transplantation experiments. CTX was injected into TA muscle 1 day before transplantation. Then, GFP(+) myoblasts (Mb) alone or with a mixture of GFP(+) myoblasts and CD31(−) CD45(−) SP cells derived from wild-type (WT) mice were transplanted to CTX-injected TA muscles of 8- to 12-week-old *NOD/scid* mice, and sampled 2 weeks after transplantation. **B:** Cross-sections of transplanted TA muscles stained with anti-GFP (green) and anti-laminin-α2 chain (red) antibodies. Nuclei were stained with TOTO3 (blue). **C:** The number of GFP(+) fibers per cross section of transplanted TA muscle. Values are means with SE (seven to eight mice in each group). $^{**}P < 0.01$. **D:** Average diameters of GFP(+) fibers in the TA muscles transplanted with myoblasts (Mb) or myoblasts plus CD31(−) CD45(−) SP cells (Mb + SP). Values are means with SE. $^{***}P < 0.001$. Scale bar = 80 μm.

The transplantation efficiency of myoblasts in *mdx* mice was 40 to 60% lower than that in *NOD/scid* mice. In the present study, *mdx* mice were not treated with any immunosuppressant. Although cellular infiltration was not evident when examined 2 weeks after transplantation (data not shown), some immune reaction might be evoked and eliminate myoblasts transplanted into *mdx* muscle.

Localization of Transplanted Myoblasts and CD31(−) CD45(−) SP Cells after Intramuscular Injection

To examine the interaction between grafted myoblasts and CD31(−) CD45(−) SP cells during muscle regeneration, we labeled C57BL/6 myoblasts with a retrovirus vector expressing a red fluorescent protein, DsRed. CD31(−) CD45(−) SP cells were isolated from GFP-Tg mice. We then injected a mixture of DsRed(+) myoblasts and GFP(+) CD31(−) CD45(−) SP cells into CTX-injected *NOD/scid* TA muscles. At 24 hours after transplantation, DsRed(+) myoblasts and GFP(+) CD31(−) CD45(−) SP cells were observed clearly (Figure 4A). At 48 hours after transplantation, immunohistochemistry revealed that grafted CD31(−) CD45(−) SP cells expanded, and surrounded both grafted myoblasts and damaged myofibers, but rarely fused with myoblasts (Figure 4B).

CD31(−) CD45(−) SP Cells Promote Proliferation of Myoblasts in Vivo *and* in Vitro

Next, to clarify the mechanism by which co-transplanted CD31(−) CD45(−) SP cells increased the contribution of grafted myoblasts to myofiber regeneration, we investigated the survival of grafted myoblasts after transplantation (Figure 5). GFP(+) myoblasts were injected into TA muscles of *NOD/scid* mice with or without unlabeled CD31(−) CD45(−) SP cells. At 24, 48, and 72 hours after transplantation, injected TA muscles were dissected, and the GFP mRNA level in injected muscles was evaluated by using real-time PCR (Figure 5A). There was a decline of the GFP mRNA level of injected muscles from 24 to 72 hours after injection (Figure 5B) with no differences in survival rates between single transplantation and co-transplantation.

At 48 and 72 hours after transplantation, however, GFP mRNA levels were slightly higher in co-injected muscle than in muscle injected with myoblasts alone (Figure 5B). Therefore, we directly counted the number of GFP(+) myoblasts at 72 hours after transplantation. As shown in Figure 6, A and B, many more GFP(+) myoblasts were detected in co-transplanted muscles than in myoblast-transplanted muscles (Figure 6, A and B). In addition, GFP(+) cells were more widely spread in the co-injected muscles than in muscles transplanted with myoblasts alone (Figure 6C).

To determine whether CD31(−) CD45(−) SP cells promote proliferation of implanted myoblasts, we dissected the muscles at 48 hours after transplantation, and stained the cross-sections with anti-phosphorylated histone H3 antibody, a marker of the mitotic phase of the cell cycle. Co-transplantation of myoblasts with CD31(−) CD45(−) SP cells significantly increased the percentage of mitotic GFP(+) cells compared with transplantation of myoblasts alone (Figure 6D). These observations suggest that co-injection of CD31(−) CD45(−) SP cells promoted proliferation of grafted myoblasts.

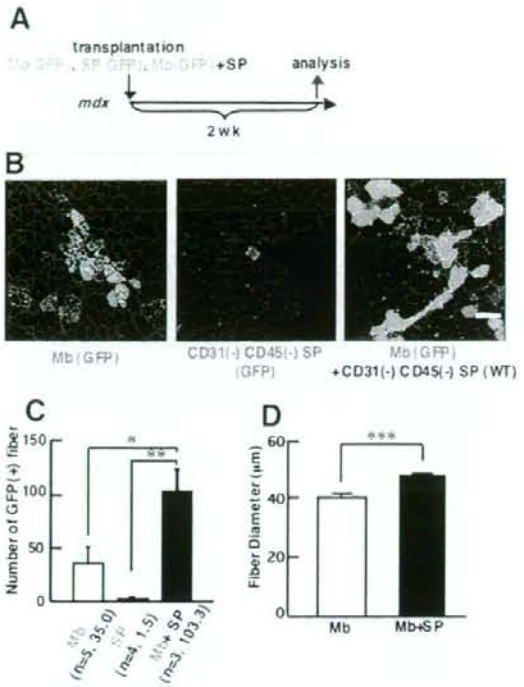

Figure 3. Co-transplantation of CD31(−) CD45(−) SP cells and myoblasts improves efficiency of myoblast transfer in dystrophin-deficient *mdx* mice. **A:** Schematic protocol of experiments. GFP(+) myoblasts alone (3×10^4), GFP(+) CD31(−) CD45(−) SP cells alone (3×10^4 cells), or a mixture of GFP(+) myoblasts (3×10^4) and CD31(−) CD45(−) SP cells (2×10^4) were directly injected into TA muscles of 8-week-old *mdx* mice, and the muscles were sampled 2 weeks after transplantation. **B:** Cross-sections of transplanted TA muscles stained with anti-GFP (green) and anti-laminin-α2 chain (red) antibodies. Nuclei were stained with TOTO3 (blue). **C:** The number of GFP(+) fibers per cross section. Myoblasts gave rise to more myofibers when co-transplanted with CD31(−) CD45(−) SP cells (Mb + SP) than when transplanted alone (Mb). Transplantation of only GFP(+) SP cells resulted in formation of few myofibers (SP). Values are means with SE ($n = 3$ to 5 mice). $^*P < 0.05$, $^{**}P < 0.01$. **D:** Average diameters of GFP(+) fibers in the TA muscles transplanted with myoblasts (Mb) or with myoblasts plus CD31(−) CD45(−) SP cells (Mb + SP). Values are means with SE. $^{***}P < 0.001$. Scale bar = 80 μm.

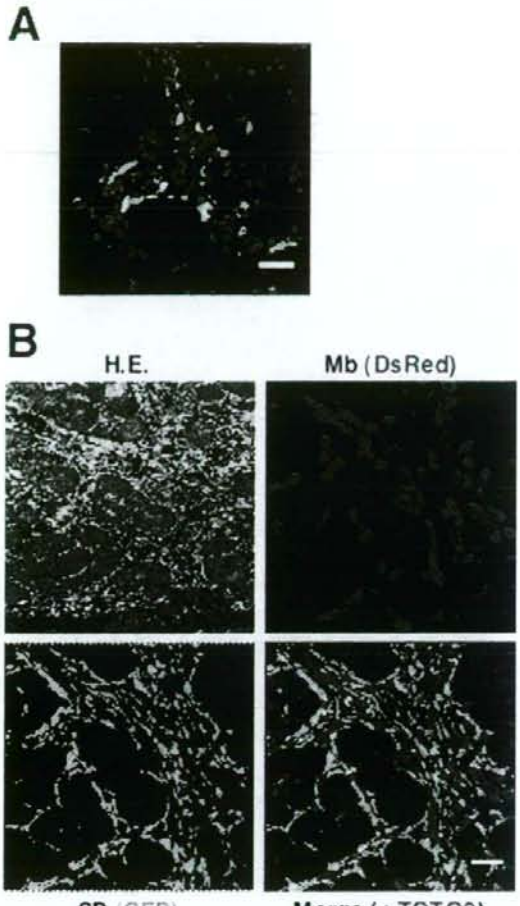

Figure 4. Behavior of GFP$^+$ CD31(−) CD45(−) SP cells and DsRed-labeled myoblasts after transplantation. **A:** *NOD/scid* TA muscles were injected with CTX 24 hours before transplantation. Then, myoblasts transduced with a retrovirus vector expressing DsRed were injected together with GFP(+) CD31(−) CD45(−) SP cells into the muscles. The muscles were dissected 24 hours after the transplantation, sectioned, and stained with anti-DsRed (red) and anti-GFP antibodies (green). Nuclei were stained with TOTO3 (blue). **B:** Representative image of DsRed(+) myoblasts and GFP(+) SP cells 48 hours after co-transplantation. One serial section was stained with H&E. Scale bars = 40 μm.

Next, to examine whether CD31(−) CD45(−) SP cells directly promote proliferation of myoblasts or not, we performed an *in vitro* proliferation assay using primary myoblasts and conditioned medium (CM) of CD31(−) CD45(−) SP cells and CM of 10T1/2 cells. BrdU uptake analysis showed that SP-CM more strongly stimulated the proliferation of myoblasts than 10T1/2-CM did (Figure 6E). The results suggest that CD31(−) CD45(−) SP cells promote proliferation of injected myoblasts at least in part by producing soluble factors.

Gene Expression Profiling of CD31(−) CD45(−) SP Cells

To identify the growth factor produced by CD31(−) CD45(−) SP cells that promotes proliferation of myoblasts, we extracted total RNAs from CD31(−) CD45(−) SP cells, myoblasts, and macrophages isolated from regenerating muscles 3 days after CTX injection, and examined the gene expression in these three cell populations by microarray. Eventually, we identified 192 genes that were expressed at more than 10-fold higher levels in CD31(−) CD45(−) SP cells than in either macrophages or myoblasts. We categorized the 192 genes based on gene ontology, and found that CD31(−) CD45(−) SP cells preferentially express extracellular matrix proteins and cytokines and their receptors (see Supplementary Table S1 at *http://ajp.amjpathol.org*). We found numerous genes involved in wound healing and tissue repair on the gene list, suggesting that CD31(−) CD45(−) SP cells play a regulatory role in the muscle regeneration process. Interestingly, the gene list contained both muscle prolif-

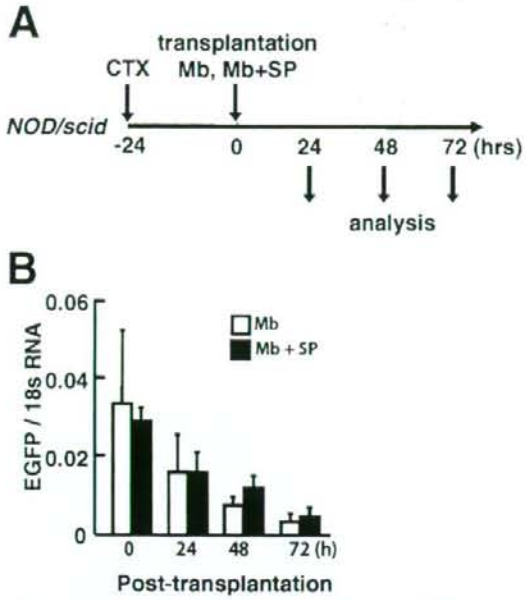

Figure 5. Survival of injected myoblasts in *NOD/scid* mice. **A:** Experimental design. GFP(+) myoblasts alone (3×10^4 cells) or a mixture of GFP(+) myoblasts (3×10^4 cells) and nonlabeled CD31(−) CD45(−) SP cells (2×10^4 cells) were injected into previously CTX-injected TA muscles of *NOD/scid* mice. The muscles were then sampled at 0, 24, 48, and 72 hours after transplantation. **B:** The mRNA level of GFP at each time point was quantified by real-time PCR. The *y* axis shows GFP mRNA levels normalized to 18s RNA with SE ($n = 4$ to 5).

eration or differentiation-promoting (follistatin),[26] and inhibitory factors (eg, insulin-like growth factor binding proteins,[27] Nov[28]). The list also contains regulators of TGF-β (eg, thrombospondins,[29] Prss11,[30] Ltbp3[31]), which would consequently attenuate or stimulate proliferation and differentiation of myoblasts.

CD31(−) CD45(−) SP Cell-Derived MMP-2 Promotes the Migration of Myoblasts

Genome-wide gene expression analysis revealed that CD31(−) CD45(−) SP cells highly express matrix metalloproteinases (see Supplementary Table S1 and Supplementary Figure S1 at *http://ajp.amjpathol.org*). MMPs are a group of zinc-dependent endopeptidases that degrade extracellular matrix components, thereby facilitating cell migration and tissue remodeling.[32,33] Furthermore, MMPs are known to release growth factors stored within the extracellular matrix and process growth factor receptors, resulting in stimulation of cell proliferation.[34–36] Among the MMPs up-regulated in CD31(−) CD45(−) SP cells, we paid special attention to MMP-2 (also called gelatinase A or 72-kDa type IV collagenase). In CTX-injected muscle, MMP-2 activity was shown to be increased concomitantly with the transition from the regeneration phases characterized by the appearance of young myotubes to maturation of the myotubes into multinucleated myofibers[37,38] MMP-2 was also activated in the endomysium of regenerating fibers in dystrophin-deficient muscular dystrophy dogs.[39] Furthermore, MMP-2 transcripts were found in the areas of fiber regeneration, and were localized to mesenchymal fibroblasts in DMD skeletal muscle.[40]

We confirmed that the mRNA level of MMP-2 was much higher in CD31(−) CD45(−) SP cells than in macrophages or myoblasts (Figure 7A). Next, we examined the gelatinolytic activity in CD31(−) CD45(−) SP cells, macrophages, and myoblasts by DQ-gelatin zymography. The cells were directly isolated from regenerating muscle. High gelatinolytic activity was detected in CD31(−) CD45(−) SP cells, compared to myoblasts or macrophages (Figure 7B). Importantly, the signal in MMP-2-null SP cells was considerably weak, compared with wild-type SP cells. The results indicate that DQ-gelatin was degraded mainly (but not exclusively) by MMP-2 in the assay. We hardly detected the green fluorescence in wild-type SP cells in the presence of a broad-spectrum inhibitor of MMPs, GM6001, but not a potent inhibitor of cysteine proteases, E-64, suggesting that other MMPs contribute to gelatin degradation to some extent in the assay. Collectively, these results indicate that CD31(−) CD45(−) SP cells have high MMP-2 activity.

MMP-2 is reported to mediate cell migration and tissue remodeling.[32,33] To directly investigate the effects of MMP-2 on the migration and proliferation of transplanted myoblasts, we injected GFP(+) myoblasts with CD31(−) CD45(−) SP cells prepared from wild-type mice or from MMP-2-null mice into CTX-injected TA muscles of *NOD/scid* mice. There was no difference in the yield of CD31(−) CD45(−) SP cells from regenerating muscle between wild-type and MMP-2-null mice (data not shown). Consistent with this observation, MMP-2-null CD31(−) CD45(−) SP cells proliferated as vigorously as wild-type *in vitro* (data not shown). At 72 hours after transplantation, GFP(+) myoblasts were more widely spread in the muscle co-injected with wild-type CD31(−) CD45(−) SP cells than in the muscles co-injected with MMP-2-deficient CD31(−) CD45(−) SP cells (Figure 7C). In contrast, there was no difference in the number of GFP(+) myoblasts between two groups (Figure 7D). These results strongly suggest that MMP-2 derived from CD31(−) CD45(−) SP cells significantly promotes migration of myoblasts, but does not influence the proliferation of myoblasts.

Discussion

We previously reported a novel SP subset: CD31(−) CD45(−) SP cells.[20] They are resident in skeletal muscle and are activated and vigorously proliferate during muscle regeneration. RT-PCR analysis suggested that CD31(−) CD45(−) SP cells are of mesenchymal lineage, and indeed they differentiated into adipocytes, osteogenic cells, and muscle cells after specific induction *in vitro*.[20] In the present study, we further characterized CD31(−) CD45(−) SP cells and found that co-transplantation of CD31(−) CD45(−) SP cells markedly improves the efficacy of myoblast transfer to dystrophic *mdx* mice. Our

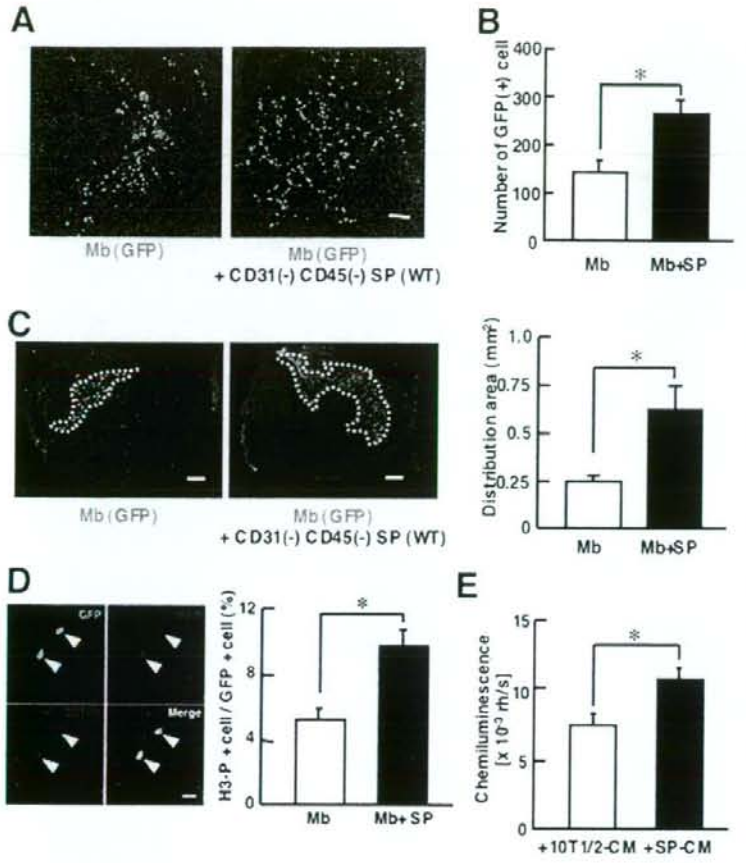

Figure 6. CD31(−) CD45(−) SP cells promote proliferation of myoblasts *in vitro* and *in vivo*. **A:** Representative images of cross sections of 72-hour samples stained with anti-GFP (green) and anti-laminin-α2 chain (red) antibodies. GFP(+) myoblasts are more widely scattered in injected muscle when co-transplanted with CD31(−) CD45(−) SP cells, compared with single transplantation. **B:** The number of GFP(+) cells per cross section of TA muscles injected with myoblasts or myoblasts and CD31(−) CD45(−) SP cells. Values were means with SE (n = 4 to 5). $^*P < 0.05$. **C: Left:** Representative distributions of GFP(+) myoblasts-myotubes 72 hours after transplantation. **Right:** Distribution area (marked by white dotted lines in **left** panels) was measured by Image J software. Values were means with SE (n = 4 to 5). $^*P < 0.05$. **D:** GFP(+) myoblasts were transplanted into CTX-injected TA muscles of *NOD/scid* mice with (Mb + SP) or without CD31(−) CD45(−) SP cells (Mb). Forty-eight hours after transplantation, the muscles were dissected, sectioned, and stained with anti-phosphorylated histone-H3 (H3-P) (red) and anti-GFP (green) antibodies. **Arrowheads** indicate H3-P(+) GFP(+) cells. The **right** graph shows the percentage of H3-P(+) cells in GFP(+) myoblasts in single-transplanted muscle (Mb) or in co-transplanted muscle (Mb + SP). The values are means with SE (n = 3). $^*P < 0.05$. **E:** Myoblasts were cultured for 3 days in conditioned medium of either CD31(−) CD45(−) SP cells (SP-CM) or 10T1/2 cells (10T1/2-CM) and then cultured for an additional 24 hours in the presence of BrdU. The vertical axis shows BrdU uptake by myoblasts. Values are means with SE (n = 6). $^*P < 0.05$. Scale bars: 100 μm (**A**); 200 μm (**C**); 80 μm (**D**).

findings suggest that endogenous CD31(−) CD45(−) SP cells support muscle regeneration by stimulating proliferation and migration of myoblasts.

Are CD31(−) CD45(−) SP Cells Mesenchymal Stem Cells?

Analysis of cell surface antigens on CD31(−) CD45(−) SP cells suggests that they are a homogeneous population. Several reports showed that mesenchymal stem cells (MSCs) express CD44, CD90, but not CD31, CD45, or CD14.[41,42] The expression patterns of these markers on CD31(−) CD45(−) SP cells and their differentiation potentials into osteogenic cells, adipocytes, and myogenic cells suggest that CD31(−) CD45(−) SP cells are closely related to MSCs.[20] On the other hand, the expression of PDGFRβ,[20] CD44, CD49b, CD90, and the lack of CD133 expression on CD31(−) CD45(−) SP cells are similar to those of human pericytes.[13] Unlike human pericytes, however, CD31(−) CD45(−) SP cells have limited myogenic potential *in vivo*.[13,20] The relationship between CD31(−) CD45(−) SP cells and MSCs or pericytes remains to be determined in a future study.

CD31(−) CD45(−) SP Cells Promote Proliferation of Myogenic Cells

In the present study, we demonstrated that the efficiency of myoblast transfer is greatly improved by co-transplantation of CD31(−) CD45(−) SP cells. Transplanted CD31(−) CD45(−) SP cells proliferated in the injection site and surrounded both engrafted myoblasts and damaged myofibers, but rarely fused with myoblasts (Figure 4). Transplantation of CD31(−) CD45(−) SP cells alone contributed little to myofiber formation. Therefore, the improvement in efficiency of myoblast transfer by co-transplantation is not attributable to differentiation of CD31(−) CD45(−) SP cells into muscle fibers.

Because the conditioned medium from CD31(−) CD45(−) SP cells modestly stimulated the proliferation of myoblasts *in vitro*, when compared with CM of 10T1/2 cells, it is possible that CD31(−) CD45(−) SP cells stimulated proliferation of myoblasts by secreting growth factors. CD31(−) CD45(−) SP cells are found in close vicinity to myoblasts 48 hours after transplantation. Therefore, even low levels of growth factors produced by CD31(−) CD45(−) SP cells may effectively stimulate the prolifera-

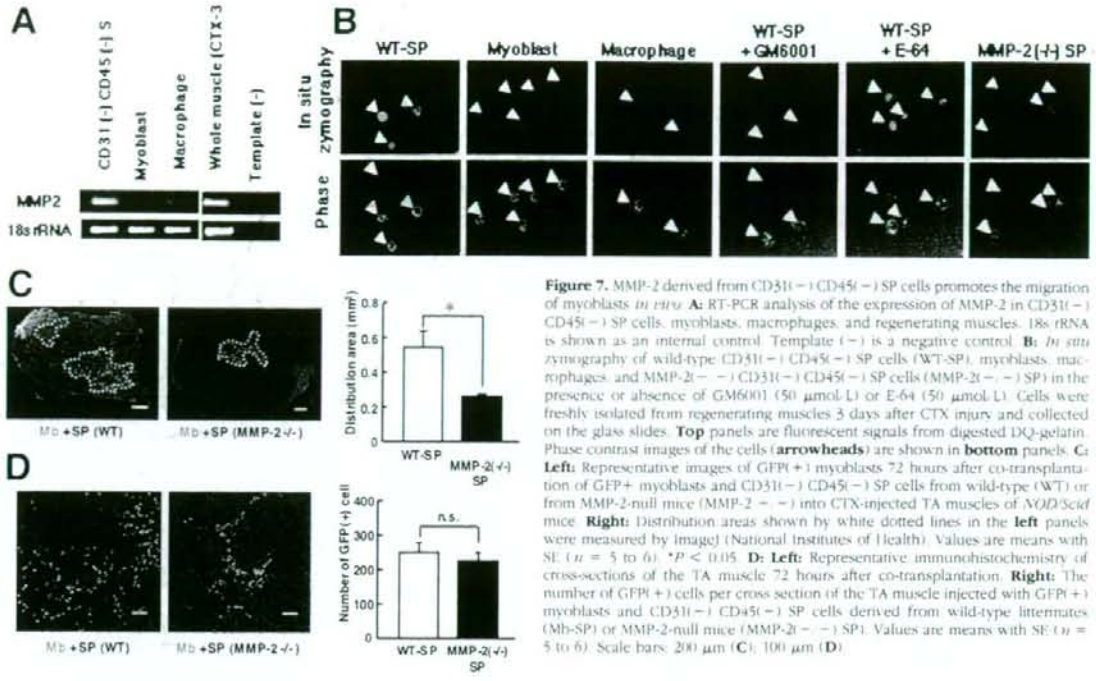

Figure 7. MMP-2 derived from CD31(−) CD45(−) SP cells promotes the migration of myoblasts *in vitro*. **A:** RT-PCR analysis of the expression of MMP-2 in CD31(−) CD45(−) SP cells, myoblasts, macrophages, and regenerating muscles. 18s rRNA is shown as an internal control. Template (−) is a negative control. **B:** *In situ* zymography of wild-type CD31(−) CD45(−) SP cells (WT-SP), myoblasts, macrophages, and MMP-2(−/−) CD31(−) CD45(−) SP cells (MMP-2(−/−) SP) in the presence or absence of GM6001 (50 μmol·L) or E-64 (50 μmol·L). Cells were freshly isolated from regenerating muscles 3 days after CTX injury and collected on the glass slides. **Top** panels are fluorescent signals from digested DQ-gelatin. Phase contrast images of the cells (**arrowheads**) are shown in **bottom** panels. **C:** **Left:** Representative images of GFP(+) myoblasts 72 hours after co-transplantation of GFP+ myoblasts and CD31(−) CD45(−) SP cells from wild-type (WT) or from MMP-2-null mice (MMP-2 −/−) into CTX-injected TA muscles of *NOD/Scid* mice. **Right:** Distribution areas shown by white dotted lines in the **left** panels were measured by ImageJ (National Institutes of Health). Values are means with SE (n = 5 to 6). *$P < 0.05$. **D:** **Left:** Representative immunohistochemistry of cross-sections of the TA muscle 72 hours after co-transplantation. **Right:** The number of GFP(+) cells per cross section of the TA muscle injected with GFP(+) myoblasts and CD31(−) CD45(−) SP cells derived from wild-type littermates (Mb+SP) or MMP-2-null mice (MMP-2(−/−) SP). Values are means with SE (n = 5 to 6). Scale bars: 200 μm (**C**); 100 μm (**D**).

tion of myoblasts. Importantly, several reports showed that MSCs secrete a variety of cytokines and growth factors, which suppress the local immune system, inhibit fibrosis and apoptosis, enhance angiogenesis, and stimulate mitosis and differentiation of tissue-specific stem cells.[43] On the gene list, we found a variety of cytokines/chemokines and their regulators (see Supplementary Table S1 at *http://ajp.amjpathol.org*). These molecules may directly or indirectly stimulate proliferation of myoblasts.

MMP-2 Derived from CD31(−) CD45(−) SP Cells Promotes the Migration of Myoblasts

Transplanted GFP(+) myoblasts were more widely spread in injected muscle when co-injected with CD31(−) CD45(−) SP cells than when transplanted alone (Figure 6C). MMP-2 is a candidate molecule that promotes migration of myoblasts. MMP-2 plays a critical role in myogenesis[44] and is up-regulated in muscle regeneration (see Supplementary Figure S2 at *http://ajp.amjpathol.org*).[38] MMP-2 expression is also detected in regenerating areas of dystrophic muscles.[39,40] Importantly, El Fahime and colleagues[45] reported that forced expression of MMP-2 in normal myoblasts significantly increased migration of myoblasts *in vivo*. In the present study, we demonstrated that CD31(−) CD45(−) SP cells highly express MMP-2 (see Figure 7A and Supplementary Table S1 at *http://ajp.amjpathol.org*). Gelatin zymography confirmed that CD31(−) CD45(−) SP cells have high gelatinolytic activities (Figure 7B). Importantly, CD31(−) CD45(−) SP cells prepared from wild-type mice promoted the migration of transplanted myoblasts, but those from MMP-2-null mice did not (Figure 7C). Our results suggest that CD31(−) CD45(−) SP cells promote the migration of myoblasts via MMP-2 secretion. CD31(−) CD45(−) SP cells highly express MMP-2, 3, 9, 14, and 23 during regenerating muscle (see Supplementary Figures S1 and S2 and Supplementary Table S1 at *http://ajp.amjpathol.org*). Therefore, it remains to be determined whether MMPs other than MMP-2 also promote the migration of myoblasts. MMPs are reported to promote cell proliferation by releasing local growth factors stored within the extracellular matrix and process growth factor receptors.[34,35,46] In the present study, however, MMP-2 derived from CD31(−) CD45(−) SP cells did not stimulate the proliferation of myoblasts *in vivo* (Figure 7D). The factors that stimulate the proliferation of myoblasts remain to be determined in a future study. MMP-3, -9, -14, and -23 are candidates that play a role in stimulating the proliferation of myoblasts.

CD31(−) CD45(−) SP Cells Are the Third Cellular Component of Muscle Regeneration

Our results suggest that transplanted CD31(−) CD45(−) SP cells stimulate myogenesis of co-transplanted myoblasts by supporting their proliferation and migration. Our results also suggest that endogenous CD31(−) CD45(−) SP cells promote muscle regeneration by the same mechanisms. Muscle regeneration is a complex, highly coordinated process in which not only myogenic cells but also inflammatory cells such as macrophages play critical roles.[3] Based on our finding that CD31(−) CD45(−) SP cells regulate myoblast proliferation and migration, we

propose that CD31(−) CD45(−) SP cells are a third cellular component of muscle regeneration. In addition, gene expression analysis on CD31(−) CD45(−) SP cells revealed that CD31(−) CD45(−) SP cells express a wide range of regulatory molecules implicated in embryonic development, tissue growth and repair, angiogenesis, and tumor progression, suggesting that CD31(−) CD45(−) SP cells are a versatile player in regeneration of skeletal muscle. Future studies of ablation of endogenous CD31(−) CD45(−) SP cells in the mouse will likely further clarify the mechanisms by which CD31(−) CD45(−) SP cells promote muscle regeneration.

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Research Article

Suppression of macrophage functions impairs skeletal muscle regeneration with severe fibrosis

Masashi Segawa[a], *So-ichiro Fukada*[a,*], *Yukiko Yamamoto*[a], *Hiroshi Yahagi*[a], *Masanori Kanematsu*[a], *Masaki Sato*[a], *Takahito Ito*[a], *Akiyoshi Uezumi*[b], *Shin'ichi Hayashi*[c], *Yuko Miyagoe-Suzuki*[d], *Shin'ichi Takeda*[d], *Kazutake Tsujikawa*[a], *Hiroshi Yamamoto*[a]

[a]*Department of Immunology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, 1-6 Yamada-oka, Osaka 565-0871, Japan*
[b]*Division for Therapies Against Intractable Diseases, Institute for Comprehensive Medical Science, Fujita Health University, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan*
[c]*Department of Molecular and Cellular Biology, School of Life Science, Faculty of Medicine, Tottori University, Yonago, Tottori 683-8503, Japan*
[d]*Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan*

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ABSTRACT

When damaged, skeletal muscle regenerates. In the early phases of regeneration, inflammatory cells such as neutrophils/granulocytes and macrophages infiltrate damaged muscle tissue. To reveal the roles of macrophages during skeletal muscle regeneration, we injected an antibody, AFS98 that blocks the binding of M-CSF to its receptor into normal mice that received muscle damages. Anti-M-CSF receptor administration suppressed macrophage but not neutrophil infiltration. Histological study indicated that suppression of macrophages function leads to the incomplete muscle regeneration. In addition FACS and immunohistochemical study showed that the acute lack of macrophages delayed proliferation and differentiation of muscle satellite cells *in vivo*. Furthermore, mice injected with the anti-M-CSF receptor antibody exhibited not only adipogenesis, but also significant collagen deposition, i.e., fibrosis and continuous high expression of connective tissue growth factor. Finally we indicate that these fibrosis markers were strongly enriched in CD90(+) cells that do not include myogenic cells. These results indicate that macrophages directly affect satellite cell proliferation and that a macrophage deficiency severely impairs skeletal muscle regeneration and causes fibrosis.

Introduction

Skeletal muscle has the ability to regenerate after damage. Postnatal growth and repair of skeletal muscle are dependent on muscle satellite cells located between the basal lamina and sarcolemma of myofibers [1,2]. The muscle satellite cell is known to be a key player in skeletal muscle regeneration [3–5]. In addition, when muscle is damaged physically or chemically, subsequent cellular responses, such as infiltration by inflammatory cells, are observed. As in other inflammatory reactions, neutrophils/

* *Corresponding author.* Fax: +81 6 6879 8194.
E-mail address: fukada@phs.osaka-u.ac.jp (S. Fukada).

Abbreviations: CTGF, connective tissue growth factor; CTX, cardiotoxin; mAb, monoclonal antibody; M-CSF, macrophage colony-stimulating factor; M-CSFR, macrophage colony-stimulating factor receptor; TA, tibialis anterior; TGF-β, tumor growth factor beta

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granulocytes (polymorphonuclear leukocytes) infiltrate rapidly, followed by macrophage infiltration [6]. In particular, macrophages are thought to regulate the proliferation and differentiation of satellite cells through the production of several growth factors [7] because the macrophage invasion is coincident with the start of satellite cell proliferation and muscle regeneration.

Previously, it was reported that the proliferation of and myotube formation by rat myoblasts or mouse C2C12 cells were accelerated by the presence of the culture supernatant of peritoneal macrophages or co-cultured with them [8,9]. Recently, a factor released by macrophages, TWEAK, was shown to be a proliferation factor for myogenic cells and an inhibitor of myogenic differentiation [10,11]. Hirata et al. reported that the expression of many genes was significantly higher during cardiotoxin-induced muscle regeneration [12]. Among them, the release of osteopontin, a versatile regulator of tissue repair, by macrophages was up-regulated in regenerating muscles at 48 h after injury. These results suggest that macrophages not only clean up debris but also release many cytokines or chemokines during skeletal muscle regeneration.

Several studies have examined the *in vivo* roles of macrophages in skeletal muscle regeneration. Lescaudron et al. indicated that inhibition of hematopoietic cells including macrophage proliferation by irradiation leads to slow muscle repair [13]. Charo and Taubman reported that monocyte chemoattractant protein (MCP)-1/CCR2 (known as an MCP-1 receptor) signaling is critical for macrophage invasion [14]. Shireman et al. [30] showed that MCP-1 is necessary for skeletal muscle regeneration by excising femoral arteries in MCP-1-deficient mice. In addition, the presence of the MCP-1 receptor CCR2 is necessary for skeletal muscle regeneration *in vivo*, and fat accumulation was observed in CCR2-null mice, followed by decreased macrophage invasion into regenerating muscle, although CCR2 was expressed on myogenin-positive myogenic cells [15]. Furthermore, recently Arnold et al. [33] reported interesting results on monocyte/macrophage heterogeneity during skeletal muscle regeneration. They also showed the *in vivo* roles of macrophages using a CD11b-diphtheria toxin, although the CD11b antigen is also expressed in neutrophils. Thus, whether specific depletion of macrophages affects satellite proliferation *in vivo* is still unknown.

In this study, we examined the roles of macrophages in skeletal muscle regeneration using the injection of anti-M-CSFR-monoclonal antibody (AFS98) into normal mice. Using this model, we found that specific inhibition of macrophage infiltration was associated with unusual skeletal muscle regeneration. We also found a delay in the proliferation and differentiation of satellite cells and severe fibrosis in the AFS98-treated mice. Moreover several fibrosis-related molecules, collagen I, connective tissue growth factor (CTGF), and tumor growth factor beta 3 (TFGβ3), were enriched in CD90(+) cells that are non-myogenic and -hematopoietic cells reported previously [16]. Therefore, our findings reveal that macrophages are necessary for efficient proliferation and differentiation of satellite cells *in vivo*, and that a lack of macrophages leads to muscle fibrosis.

Materials and methods

Animals

The mice used in the present study were maintained in the specific pathogen-free condition in our animal facility. C3H/HeN aged 6 to 8 weeks were purchased from Charles River Japan (Yokohama, Japan). All procedures used on experimental animals were approved by the Experimental Animal Care and Use Committee at the Graduate School of Pharmaceutical Sciences, Osaka University.

Antibodies

A rat monoclonal antibody to mouse M-CSFR (AFS98, IgG2a) [17] was a gift from Dr. S.-I. Nishikawa (RIKEN, Kobe, Japan). Anti-Gr-1 (clone RB6-8C5), anti-CD31 (clone 390), anti-CD45 (clone 30-F11), anti-Sca-1 (clone 7D), and Ki-67 (clone B56) antibodies were purchased from BD Pharmingen (San Diego, CA). Biotinylated rat anti-F4/80 (clone CI: A3-1) and PE-conjugated rat anti-CD90 (clone CT-TH1) were purchased from Caltag Laboratories (Burlingame, CA). A rat monoclonal antibody to mouse CD4 (clone GK1.5, IgG2b) was a gift of Dr. H. Ishikawa (Keio University, Tokyo, Japan). An SM/C-2.6 mAb to muscle satellite cells was prepared and biotinylated in our laboratories [18,19]. Rabbit anti-M-cadherin antibody was prepared as previously described [20]. Mouse anti-embryonic myosin heavy chain (eMyHC clone F1.652) antibody was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). Antibody against collagen type I was purchased from Biogenesis (England, UK).

Muscle regeneration

Muscle regeneration was induced by injecting 75 μl ardiotoxin (CTX; 10 μM, Latoxan, Rosans, France) into the bilateral tibialis anterior (TA) muscle according to methods described previously [21,22].

Immunohistological studies (H.E., Oil red O, and Masson trichrome staining)

TA muscles were isolated, frozen in liquid nitrogen-cooled isopentane, and cryosections were examined histologically. Sections (10 μm) were stained with hematoxylin–eosin and Oil red O (Sigma-Aldrich, St. Louis, MO). To visualize fibrosis in the muscle sections, some were examined by Masson trichrome staining. The signals were recorded photographically using an Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Immunohistochemical study (fluorescent staining)

Immunofluorescent staining of muscle cryosections was performed as previously described [22]. Gr-1 staining was examined using a FITC-conjugated antibody. F4/80 staining was performed using biotin-conjugated rat anti-F4/80, and sections were then reacted with streptoavidin-rhodamine (Molecular Probes, Eugene, OR). For M-CSF receptor staining, sections were performed using AFS98 mAb, and sections were then stained with rhodamine-conjugated goat anti-rat IgG (Chemicon International Inc., Temecula, CA). For M-cadherin and collagen type I staining, sections were reacted with Alexa 488 or Rhodamine Red-X-conjugated goat anti-rabbit antibodies (Molecular Probes). For Ki-67 and eMyHC staining, sections were stained with rhodamine-conjugated goat anti-mouse IgG antibodies (Chemicon International Inc., Temecula, CA). For Ki-67 and eMyHC staining, an M.O.M. kit™ (Vector Laboratories) was used to block endogenous mouse IgG. Sections were examined under a confocal laser-scanning microscope (model MRC1024ES, Bio-Rad Laboratories, Hercules, CA) or an Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Antibody administration

AFS98 (rat IgG2a) [23] and anti-CD4 (rat IgG2b) mAbs (4 mg/0.4 ml in PBS) or PBS alone were injected intraperitoneally into normal C3H/HeN mice 3 times at 2-day intervals. At the third injection, mice received an intramuscular injection of CTX to induce muscle regeneration. Antibody administrations were continued at two-day intervals until the mice were sacrificed.

FACS analysis

Mononuclear cells from limb muscles of 8- to 12-week-old C3H/HeN mice were prepared as described [22], and incubated on ice for 30 min in the presence of each antibody. The antibodies used to identify various cells were biotinylated-anti-F4/80 and FITC-anti-CD45 for macrophages; FITC-anti-CD45 and PE-anti-Gr-1 for neutrophils; and a cocktail of biotinylated-SM/C-2.6, FITC-anti-CD31, FITC-anti-CD45, and FITC-anti-Sca-1 (clone: 7D) antibodies for the satellite cells/myogenic fraction. CD90(+) CD31(−) CD45(−) cells were detected using PE-anti-CD90, FITC-anti-CD31, and FITC-anti-CD45 antibodies. PE-conjugated streptoavidin was used to detect the biotinylated antibodies. Flow cytometric profiles were analyzed with a FACSCalibur analyzer and CELLQuest software (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Dead cells and debris were excluded by PI-staining (Supplemental Fig. 2).

For detecting of M-CSFR on myogenic cells, aliquots of AFS98 were labeled with phycoerythrin (PE) in our laboratories using an R-Phycoerythrin Labeling Kit-SH (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). To examine M-CSFR expression on a myogenic cell line (C2/4) and primary myoblasts, about 2×10^5 cells were incubated with PE-conjugated AFS98 and anti-M-cadherin antibodies in PBS containing 2% fetal calf serum (FCS, Boehringer Mannheim, Acton, MA) and 0.1% NaN_3 for 30 min on ice. Alexa 488-conjugated goat anti-rabbit antibodies were used to detect M-cadherin expression. PECs (peritoneal exudate cells) were used as a positive control for the M-CSF receptor staining, and cells were double stained with anti-F4/80 antibody.

Quantification of macrophages, neutrophil, myogenic cells, and CD90(+) CD31(−) CD45(−) cells by FACS

Dead cells and debris were gated out using PI-staining and the percentage of macrophage (F4/80(+) CD45(+)), neutrophil (Gr-1(+) CD45(+)), myogenic cells (SM/C-2.6(+) CD31(−) CD45(−) Sca-1(−)), and CD90(+) CD31(−) CD45(−) cells are calculated by FACS (Supplemental Fig. 2). Each absolute cell number was calculated by multiplying the total cell number obtained (Fig. 2B) by the percentage of each fraction.

Satellite cell culture

Satellite cells were isolated from intact adult skeletal muscle using biotinylated-SM/C-2.6 and IMag methods (BD Immunocytometry Systems, Mountain View, CA) as described in the previous report [16]. Satellite cells were cultured in a growth medium (GM) of high-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Karlsruhe, Germany) containing 20% FCS (Trace Biosciences, N.S.W., Australia), 2.5 ng/ml bFGF (Invitrogen), and penicillin (100 U/ml)–streptomycin (100 μg/ml) (Gibco BRL, Gaithersburg, MD) on culture dishes coated with Matrigel™ (BD Bioscience, San Diego, CA). C2/4 cells were cultured in high-glucose DMEM containing 10% FCS and penicillin (100 U/ml)–streptomycin (100 μg/ml).

Cell proliferation assay

SM/C-2.6-positive cells (1000 cells/well) were plated in 96-well dishes. After 12 h, M-CSF was added to each well, and culture was continued. Two days after plating, BrdU (10 μM) was added to the culture. Twelve hours later, BrdU uptake was quantified by a Cell Proliferation ELISA, BrdU Kit (Roche Diagnostics, Basel, Switzerland) and a microplate reader (Model 680, Bio-Rad, Hercules, CA).

Isolation of CD90-positive cells 7 days after CTX injection

Isolated single cells were incubated on ice for 30 min in the presence of PE-conjugated anti-CD90 antibody. Cells were then incubated with anti-PE Particles-DM (BD Biosciences). Cell sorting was performed on an IMag Immunocytometry system (BD Immunocytometry Systems, Mountain View, CA) as an isolation of satellite cells. The purity of sorted cells was higher than 90%.

Quantitative RT-PCR

Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's manual. In brief, 5 μg total RNA was used for reverse transcription (RT), and the cDNA was used for each polymerase chain reaction (PCR). Real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa, Kyoto, Japan) in a final volume of 10 μL. Specific forward and reverse primers to produce approximately 100 bp amplicons for optimal amplification in real-time PCR of reverse transcribed cDNAs were 5′-TCC ACC CGA GTT ACC AA and 5′-TTA GGT GTC CGG ATG C for mouse CTGF; 5′-GCC GCA AAG AGT CTA CA and 5′-CGG GTT TCC ACG TCT CA for mouse collagen type I; and 5′-CTT TGC TGA CCT GCT GGA TTA CAT and 5′-GTC CCC CGT TGA CTG ATC ATT AC for mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT). All primer combinations were positioned to span an intron between two exons. Real-time PCR and data analysis were performed on a LightCycler quick-system 350S using LightCycler Software (Roche Diagnostics, Basel, Switzerland). Samples were amplified, and the relative gene expression levels were calculated using standard curves generated by serial dilutions of the cDNA from injured muscle tissue. The primers, which produced approximately 500 bp amplicons using the standard curves, were 5′-TCC ACC CGA GTT ACC AA and 5′-TTA GGT GTC CGG ATG C for mouse CTGF; 5′-GCC GCA AAG AGT CTA CA and 5′-CGG GTT TCC ACG TCT CA for mouse collagen type I; and: 5′-CTT TGC TGA CCT GCT GGA TTA CAT and 5′-GTC CCC CGT TGA CTG ATC ATT AC for mouse HPRT. Samples were amplified and the quantification data of mouse CTGF and collagen type I relative to the reference gene (HPRT) were generated on the basis of a mathematical model for relative quantification in real-time RT-PCR. All samples of each gene were independently analyzed at least twice.

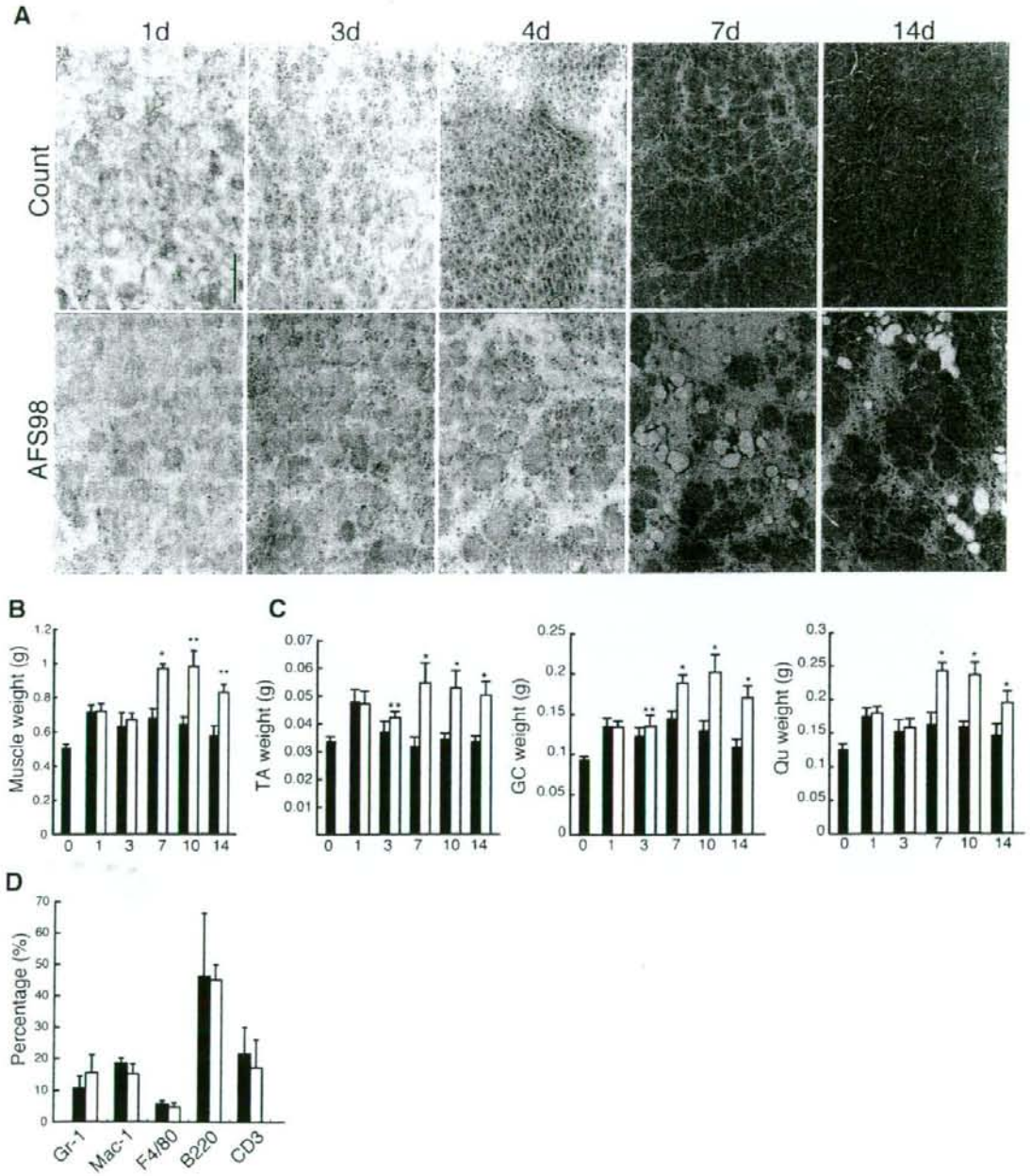


Fig. 1 – Incomplete skeletal muscle regeneration in AFS98-tretaed mice. (A) TA muscles of PBS- and AFS98-treated mice were histologically examined from 1 to 14 days after CTX injection. The cross sections were stained with hematoxylin and eosin (H.E.). Scale bar: 100 μm. (B) The total weight of right and left TA (tibialis anterior), GC (gastrocnemius), and quadriceps (Qu) muscles. Closed and open columns show PBS- and AFS98-treated mice, respectively. Results of three mice are shown. $^{*}P<0.01$, $^{**}P<0.05$ (t-test). (C) Each weight of TA, GC, and Qu muscles. $^{*}P<0.01$ (t-test). (D) The percentage of Gr-1(+), Mac-1(+), F4/80(+), B220(+), and CD3(+) splenocytes 4 days after CTX injection. Closed and open columns show PBS- ($n=3$) and AFS98-treated ($n=5$) mice, respectively.

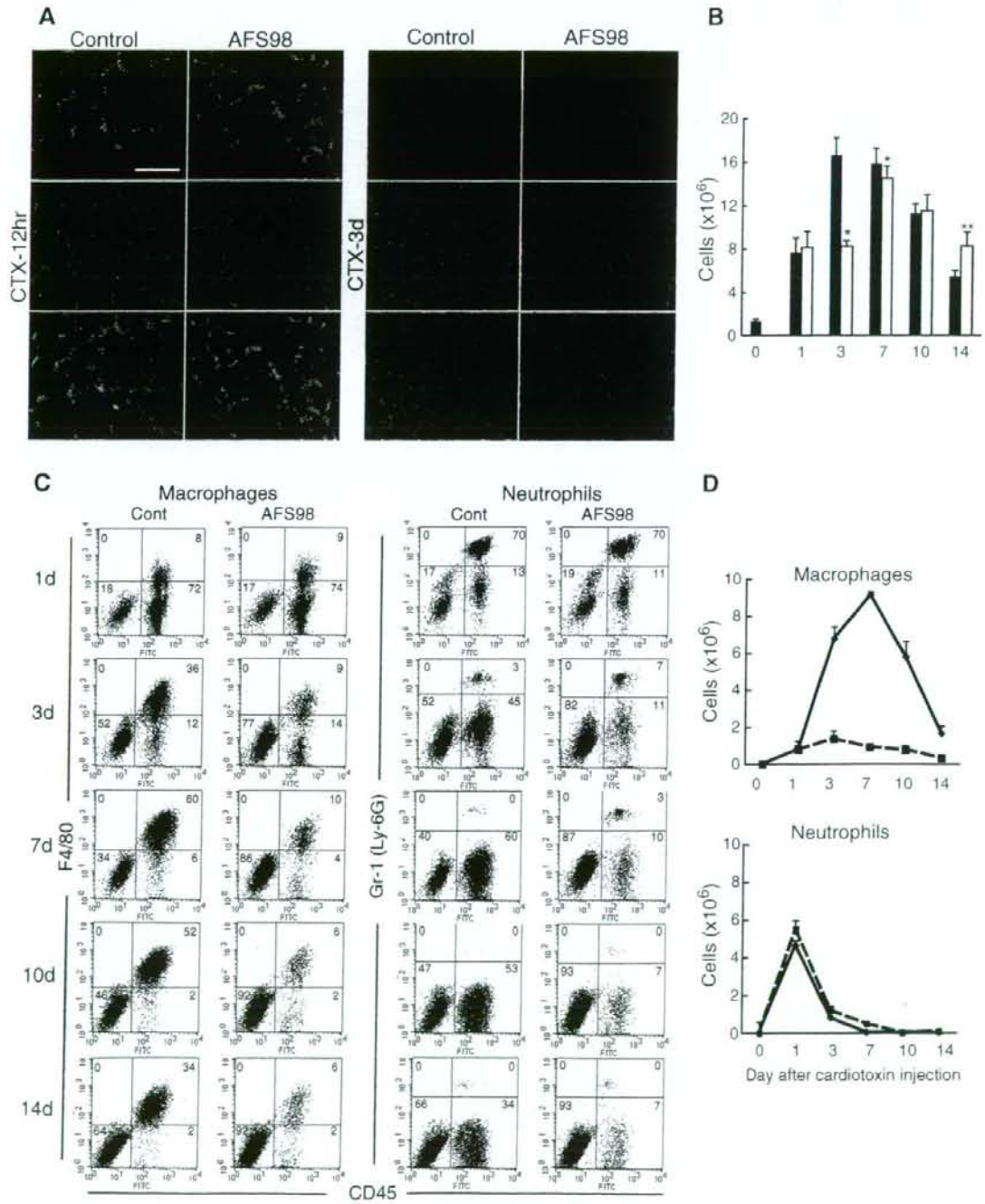
Statistics and measurement of muscle diameter

Values are expressed as means±SD. Statistical significance was assessed by unpaired Student's *t*-test. Image J software was used to measure muscle diameter, Oil red O-, and eMyHC-positive areas.

Results

Suppression of macrophage infiltration by AFS98 mAb in vivo

Macrophages may play several roles in the proliferation and differentiation of satellite cells *in vivo*. To investigate the roles of



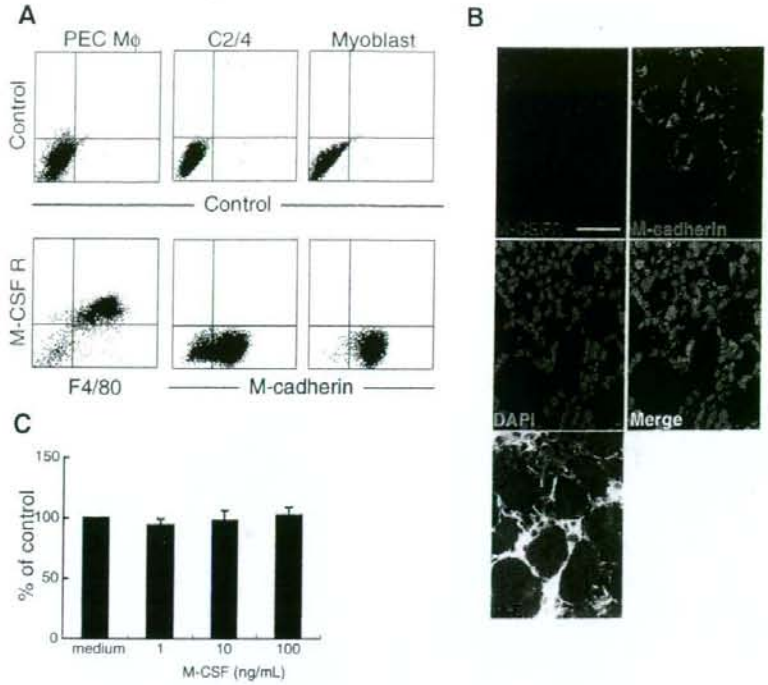


Fig. 3 – No M-CSF receptor expression in myogenic cells *in vitro* or *in vivo*. (A) C2/4 and primary mouse myoblasts were stained with anti-M-cadherin and AFS98 (M-CSFR) antibodies. The upper panels are negative controls. Macrophages in PEC were used as a positive control for the M-CSFR staining. (B) TA muscle sections 3 days after CTX injection were stained with anti-M-cadherin (green) and anti-M-CSFR (red) antibodies. Nuclei were stained with DAPI. Serial section was stained with HE. Scale bar: 100 μm. (C) BrdU uptake by primary mouse myoblasts was assayed with or without M-CSF. The vertical bars show the means with SD of three independent experiments. The result using the medium alone is shown as 100%.

macrophages in regenerating muscle, we injected an anti-M-CSFR mAb (AFS98: antagonistic anti-M-CSF receptor antibody) [17] to deplete the tissue of macrophages according to the method reported previously [23], and muscle regeneration was induced by injecting cardiotoxin (CTX). First, we histochemically examined the regeneration potential of AFS98-treated mice. As shown in Fig. 1A, incomplete muscle regeneration was observed in AFS98-treated mice. Increased muscle mass, the sign of impaired regeneration, was also observed in AFS98-treated mice (Figs. 1B and C). In spleen, all cell types were normally existed in AFS98-treated mice (Fig. 1D).

Next, we immunohistochemically investigated the cell types in both the control (PBS-injected) and AFS98-injected groups. As shown in Fig. 2A, the number of neutrophils (Gr-1$^+$) appeared to be similar to that of the control group, but macrophages (F4/80$^+$) were scarcely found in AFS98-injected groups. We also evaluated the changes in cell numbers by FACS. The total number muscle-derived mononuclear cells in AFS98-treated mice was decreased until 3 days after CTX injection; however, the number was similar to that of control mice by day 7 (Fig. 2B). On the other hand, throughout the regeneration process, macrophage infiltration was severely suppressed in AFS98-treated mice. On the 7 days after CTX injection, the average number of macrophage in control and AFS98-treated mice was $9.14 \pm 0.15 \times 10^6$ and $0.91 \pm 0.42 \times 10^6$ cells, respectively. In contrast to macrophage, there is no significant effect on neutrophil (Gr-1$^+$) infiltration (Figs. 2C and D). On the 1 day after CTX injection, the average number of neutrophils in control and AFS98-treated mice was $4.62 \pm 0.26 \times 10^6$ and 5.48 ±

Fig. 2 – (A) TA muscles from control (PBS) or AFS98-treated mice were obtained 12 h or 3 days after CTX injection. The sections were stained with anti-Gr-1 (green) or F4/80 (red) antibodies. Nuclei were stained with DAPI. Scale bar: 100 μm. (B) The total cell number of skeletal muscle-derived mononuclear cells from control (PBS) or AFS98-treated mice. Closed and open columns show PBS- and AFS98-treated mice, respectively. Results of three mice are shown. *$P<0.01$, **$P<0.05$ (t-test). (C) Representative FACS profiles of F4/80$^+$CD45$^+$ (macrophages) and Gr-1 (Ly-6G)+CD45+ fractions (neutrophils) during skeletal muscle regeneration. The number in the upper right of each FACS profile indicates the percentage of macrophages or neutrophils, respectively. (D) TA, GC, and Qu muscles of control (PBS) (solid line) and AFS98-treated (dashed line) mice were injected with CTX, and mononuclear cells from the regenerating muscles were stained with anti-F4/80 (macrophage) or Gr-1 (neutrophil) and analyzed by FACS. The horizontal axes indicate the number of days after CTX injection. The vertical axes show the mean numbers of F4/80- and Gr-1-positive cells with SD. Three mice were used for each sample.