Muscle regeneration by reconstitution with bone marrow or fetal liver cells from green fluorescent protein–gene transgenic mice

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Summary

The myogenic potential of bone marrow and fetal liver cells was examined using donor cells from green fluorescent protein (GFP)-gene transgenic mice transferred into chimeric mice. Lethally irradiated X-chromosome-linked muscular dystrophy (mdx) mice receiving bone marrow cells from the transgenic mice exhibited significant numbers of fluorescence+ and dystrophin+ muscle fibres. In order to compare the generating capacity of fetal liver cells with bone marrow cells in neonatal chimeras, these two cell types from the transgenic mice were injected into busulfan-treated normal or mdx neonatal mice, and muscular generation in the chimeras was examined. Cardiotoxin-induced (or -uninduced, for mdx recipients) muscle regeneration in chimeras also produced fluorescence+ muscle fibres. The muscle reconstitution efficiency of the bone marrow cells was almost equal to that of fetal liver cells. However, the myogenic cell frequency was higher in fetal livers than in bone marrow. Among the neonatal chimeras of normal recipients, several fibres expressed the fluorescence in the cardiotoxin-untreated muscle. Moreover, fluorescence+ mononuclear cells were observed beneath the basal lamina of the cardiotoxin-untreated muscle of chimeras, a position where satellite cells are localizing. It was also found that mononuclear fluorescence+ and desmin+ cells were observed in the explantation cultures of untreated muscles of neonatal chimeras. The fluorescence+ muscle fibres were generated in the second recipient mice receiving muscle single cells from the cardiotoxin-untreated neonatal chimeras. The results suggest that both bone marrow and fetal liver cells may have the potential to differentiate into muscle satellite cells and participate in muscle regeneration after muscle damage as well as in physiological muscle generation.

Key words: Muscle regeneration, Transplantation, Satellite cell

Introduction

During the past decade, mesenchymal stem cells in adult bone marrow, although forming a heterogeneous population, have been characterized by their ability to differentiate into various types of tissue-specific cells (Pereira et al., 1995; Prockop, 1997; Pittenger et al., 1997; Liechty et al., 2000) (reviewed by Clark and Keating, 1995; Bianco and Cossu, 1999; Bianco and Gehron-Robey, 2000). These cells produce colonies of fibroblasts, osteogenic cells or adipocytes in vitro. In vivo experiments have shown that they differentiate to form fibrous tissue, bone or cartilage when transplanted into the appropriate animal.

Wakitani et al. (Wakitani et al., 1995) were the first to show that mesenchymal stem cells from rat bone marrow have the ability to differentiate into myotubes when they are cultured in the presence of 5-azacytidine. In vivo transplantation of such cells into the muscles of X-chromosome-linked muscular dystrophy (mdx) mice induces dystrophin-positive muscle fibres (Saito et al., 1995). These results prompted investigators to speculate that the transplanted bone marrow cells can move into damaged muscle and grow into new muscle fibres in mice (for a review, see Cossu and Mavilio, 2000). This also has clinical implications, not only for muscular dystrophy but also for various tissue-specific hereditary disorders. Recently, several investigators have reported that transplanted bone marrow cells participate in the muscle regeneration process in irradiated recipient mice (Ferrari et al., 1998; Gussoni et al., 1999; Bittner et al., 1999). In these studies, bone marrow cells were distinguished from recipient cells by donor-specific gene expression signals such as nuclear β-galactosidase or the presence of a Y chromosome. However, muscle fibres containing donor-cell-derived chromosomes never exceeded 1% of the total fibres in an average muscle.

It is widely accepted that postnatal growth and repair of skeletal muscle are normally mediated by the satellite cells that surround muscle fibres. Satellite cells are mononucleate precursor cells that are located beneath the basal lamina and sarcolemma of myofibres (Mauro, 1961) (for reviews, see Bischoff, 1994; Cullen, 1997). Satellite cells first appear in the limbs of mouse embryos between embryonic day 16 and 18, and the cell number reaches a peak in neonatal mice. In adult mice, less than 5% of the total nuclei are satellite cells, and this...
gamma-ray irradiated (10 Gy) using Gamma Cell (137 Cs source, radiation chimeras. Adult (8 week old) Bone marrow or fetal liver cell reconstitution phenotype was determined by measuring serum immunoglobulin level. (C57BL/6 background, purchased from Charles River Japan). The blood was obtained and tested for chimerisms by measuring GFP+ drinking water (1 g/L) from 1 week before radiation to 2 weeks after GFP-Tg mice were injected intravenously into the recipient mice cell suspensions were prepared by flushing the marrow from the 1997). Single cell suspensions of fetal liver cells from GFP-Tg embryonic development. Gestational day 12 or 13 fetuses were obtained from pregnant normal C57BL/6 mice time-mated with GFP- Heterozygous EGFP transgenic (GFP-Tg) mice – is a useful strategy for further studies of satellite cell recruitment and muscle regeneration.

Materials and Methods

Animals and cells
Specific pathogen-free C57BL/6 mice aged between 6 to 8 weeks were purchased from Charles River Japan (Yokohama, Japan). Specific pathogen-free mdx mice (of C57BL/10 background) were provided by Central Laboratories of Experimental Animals (Kanagawa, Japan) and maintained in our animal facility by brother-sister matings. Heterozygous EGFP transgenic (GFP-Tg) mice with a C57BL/6 background (Okabe et al., 1997) were maintained in our animal facility by mating with normal C57BL/6 mice. The C57BL/6 or GFP-Tg mice were mated at night, and females were examined the next morning. The day on which a vaginal plug was found was considered day 0 of embryonic development. Gestational day 12 or 13 fetuses were obtained from pregnant normal C57BL/6 mice time-mated with GFP-Tg heterozygous male mice. GFP-Tg fetuses were identified by brief exposure to 365 nm UV light as previously described (Okabe et al., 1997). Single cell suspensions of fetal liver cells from GFP-Tg embryos were prepared by gentle teasing of the tissue. Bone marrow cell suspensions were prepared by flushing the marrow from the femora and tibiae of adult GFP-Tg mice, followed by gentle pipetting. mdx/scid mice were produced by mating mdx mice with scid mice (C57BL/6 background, purchased from Charles River Japan). The scid phenotype was determined by measuring serum immunoglobulin level.

Bone marrow or fetal liver cell reconstitution
Bone marrow chimeras were established by the usual method for radiation chimeras. Adult (8 week old) mdx mice were lethally gamma-ray irradiated (10 Gy) using Gamma Cell (137Cs source, Nuclear Canada, Ontario, Canada). 40x10^6 bone marrow cells from GFP-Tg mice were injected intravenously into the recipient mice within 3 hours after radiation. The recipient mice had received ampicillin (product of Sigma Chemicals Co., St. Louis, MO) in their drinking water (1 g/L) from 1 week before radiation to 2 weeks after radiation and reconstitution. 4 weeks after reconstitution, peripheral blood was obtained and tested for chimerisms by measuring GFP+ cells with a flowcytometer. 12 weeks after reconstitution, the muscles and lymphoid organs of the mice were examined for muscle regeneration and chimerism, respectively.

Neonatal chimeras were established as follows (Fig. 1). Time-mated pregnant C57BL/6 or mdx mice (at gestational day 17/18) received 20-40 mg/kg busulfan (Sigma Chemicals Co.) subcutaneously. Busulfan-treated neonatal mice were injected intrahepatically with either 0.1-0.2x10^6 fetal liver cells or 1.0-10x10^6 bone marrow cells from GFP-Tg mice according to the method of Yoder et al. (Yoder et al., 1997). 4 weeks after reconstitution, blood samples were collected from the tail vein and used for chimerism tests. Chimeric mice with more than 5% donor-derived GFP+ cells among their blood mononuclear cells were selected and used for further studies of muscle regeneration. 4 to 13 weeks later (age 8-17 weeks), the muscles and lymphoid organs were examined.

Histological analysis
Chimeric mice were sacrificed by cervical dislocation. Tibialis anterior (TA) and vastus lateralis (VL) muscles, or diaphragms, were isolated and frozen in liquid nitrogen-cooled isopentane. Cryosections (6 μm) were examined for GFP+ muscle fibres under a confocal laser scanning microscope (model MRC1024ES, Bio-Rad Laboratories, Hercules, CA). Excitation/emission wavelengths for GFP were 488 nm/522 nm. Sections were then stained with hematoxylin/eosin (H-E).

Immunohistochemistry
For immunohistochemical examinations, serial transverse cryosections (6 μm) or cultured tissues were stained with various antibodies. Alexa-568-conjugated monoclonal anti-dystrophin (MANDRA-1, Sigma) was a gift from M. Imamura (National Institute of Neuroscience, Tokyo, Japan). Polyclonal rabbit anti-desmin (ICN Pharmaceuticals), were used to stain muscles. The signals were recorded photographically using an Axioskop microscope (Carl Zeiss, Oberkochen, Germany) and laser scanning confocal imaging system MRC1000 (Bio-Rad Laboratories).

Test for blood chimerism
Blood samples of 4-week-old busulfan-treated neonatal chimeras were examined for chimerism. For flowcytometric analyses of chimerism, mononuclear cells from the blood, spleen and thymus of chimeras were stained with monoclonal antibodies, phycoerythrin- or biotin-conjugated anti-CD4 (RM4-5) and anti-CD8 (53-6.7) (PharMingen, San Diego, CA) for donor-derived T lymphocytes, and analyzed together with GFP and phycoerythrin-Cy5-streptavidin (Cederlane, Westbury, NY) (Kawakami et al., 1999a; Kawakami et al., 1999b). GFP fluorescence was measured at the same
Muscular regeneration in neonatal chimeras

Muscle regeneration

Selected neonatal chimeras at 4 weeks of age, with >5% donor-derived GFP+ cells, were anesthetized with sodium pentobarbital (75 mg/kg) (Abbott Laboratories, North Chicago, IL), and in some experiments (Table 1), muscle regeneration was induced by injecting 75 µl cardiotoxin (CTX; 10 mM, Latoxan, Rosans, France) into the TA muscle to necrotize the muscle according to the method of Davis et al. (Davis et al., 1993). Some mice were left CTX-untreated. Four to 13 weeks later, muscle regeneration and chimerism were examined.

Muscle fibre explantation

In order to detect muscular satellite cells in the reconstituted chimeras, muscle fibres from extensor digitorum longus (EDL) muscles from CTX-untreated neonatal chimeras were prepared and cultured essentially according to the methods of Bischoff (Bischoff, 1986) and Rosenblatt et al. (Rosenblatt et al., 1995). Briefly, dissected muscle was incubated with 0.5% type I collagenase (Worthington Biochemical, Lakewood, NJ) in Dulbecco’s modified Eagle’s medium (Gibco BRL, Grand Island, NY) at 37°C for 90 minutes. The muscle was transferred to fresh growth medium, high-glucose DMEM (Gibco BRL) containing 10% FCS (Boehringer-Mannheim GmbH, Mannheim, Germany) and penicillin (200 U/ml)/streptomycin (200 µg/ml) (Gibco BRL), and incubated in a humidified incubator at 37°C, 5% CO2 for 16 hours. The muscle mass was triturated with a fire-polished wide-mouth Pasteur pipette. Fibres were transferred to a Matrigel (Collaborative Biomedical, Bedford, MA)-coated Lab-Tek chamber (Nalge Nunc International, Naperville, IL) and cultured for 4 days. The fibres and attached cells were fixed with 4% paraformaldehyde in phosphate-buffered saline at room temperature for 10 minutes. They were then permeabilized with 0.25% Triton X-100 (Nacalai Tesque, Kyoto, Japan) at room temperature for 20 minutes, then non-specific binding was blocked by incubation with 5% skim milk (in PBS) for 10 minutes. The fixed fibres and cells were stained with anti-desmin antibody at 4°C overnight and then with rhodamine-conjugated goat anti-rabbit IgG. Samples were examined for GFP+ and/or rhodamine+ cells under a confocal laser scanning microscope. Excitation/emission wavelengths for rhodamine are 554 nm/573 nm.

Preparation of muscle-derived cells

Freshly isolated muscle-derived cells from neonatal chimeras were prepared according to the method of Rando and Blau (Rando and Blau, 1994). CTX-untreated VL muscles from neonatal chimeras were isolated, minced and digested with dispase II (2.4U/ml) (Boehringer-Mannheim) and 1% collagenase P (Boehringer-Mannheim) supplemented with CaCl2 to a final concentration of 2.5 mM. The slurry, maintained at 37°C for 45 minutes, was triturated every 15 minutes and passed through a 37 µm nylon mesh. Single cell

Fig. 2. Flow cytometric analyses of leukocyte chimerisms. (A) Splenocyte chimerisms of radiation-bone marrow chimera. (B) GFP+CD4+ and GFP+CD8+ T cells of the gated fraction of (A). (C) GFP+ cells in the peripheral blood of neonatal-bone-marrow chimera 4 weeks after reconstitution (4 weeks old). Splenic GFP+ cells (D) and splenic GFP+CD4+ and GFP+CD8+ T cells (E) of mouse C 9 weeks after reconstitution. (F) GFP+ bone marrow cells of mouse (C). (G) GFP+ cells in the peripheral blood of a neonatal-fetal liver cell chimera 4 weeks after reconstitution (4 weeks old). Thymic GFP+ cells (H), and CD4 and CD8 stainings of thymocytes (I) of mouse G at 10 weeks after reconstitution. Splenic GFP+ cells (J) and its GFP+CD4+ and GFP+CD8+ T cells (K) of mouse G. (L) GFP+ bone marrow cells of mouse G.

excitation/emission wavelength as FITC. Flow cytometric profiles were determined with a FACS Calibur analyzer and CELLQuest software (Becton Dickinson Immunocytometry Systems, Mountain View, CA).
Suspension was washed and injected into CTX-treated (24 hours before) TA muscles of mdx/scid mice. 4 weeks later, muscle sections were histologically examined.

Results
GFP+ muscle fibre generation in radiation-bone-marrow chimeras

Twelve weeks after reconstitution, splenic lymphocytes from radiation-bone-marrow chimeras were examined for GFP expression. As shown in Fig. 2A, about 85-87% of splenocytes from chimeras expressed GFP (see also Table 1). A small unidentified fraction (~13-15%), which expressed no GFP, may represent either host-derived radio-resistant cells or rapidly dividing donor cells (Kawakami et al., 1999b). CD4+ and CD8+ T cells showed normal lymphocyte development in the mice (Fig. 2B). Thymic lymphocytes and splenic B cells were also replaced by GFP+ cells at similar levels to total splenocytes (data not shown).

Several GFP+ fibres were readily identified in TA (Fig. 3A,B), VL (Fig. 3C,D) muscles and diaphragm (Fig. 3E,F) sections and are clearly distinct from surrounding GFP-negative fibres. Because one of the GFP+ fibres expressed dystrophin, as shown in Fig. 3G-I, the fibre is thought to be newly generated from donor-derived precursor cells (Fig. 3J-L; this figure shows positive control sections of normal C57BL/6 mouse as a control. Bars, 100 μm (A-F) and 50 μm (G-I).

GFP+ muscle fibre regeneration in neonatal chimeras

Lymphocyte chimerisms of neonatal chimeras were then examined. In order to enhance both tolerance and blood chimerism, mice received busulfan, an anticancer drug for lympho-myeloid neoplasms, at embryonic day 17/18, then received either adult bone marrow or fetal liver cells from GFP-Tg mice within 16 hours of birth (Fig. 1). Typical flowcytometry studies of the chimeras are shown in Fig. 2C-L. 4 weeks after neonatal injection of donor bone marrow (Fig. 2C) or fetal liver cells (Fig. 2G), the proportions of GFP+ blood leukocyte were 26% or 18%, respectively. The frequency of chimeras ranged from 1 to 64%, and mice having more than 5% donor-derived GFP+ cells in their blood leukocytes were selected. At the time of muscle examination, GFP+ splenocytes (Fig. 2D,E,J,K), thymocytes (Fig. 2H,I) and bone marrow cells (Fig. 2F,L) were also examined for chimerisms. Normal patterns of splenic mature T cells or immature thymocytes were observed. The donor-derived GFP+ cells were found to be recruited to the recipient bone marrow. The chimerisms ranged from 5 to 78% at the time when mice were killed (see Table 1).

CTX-induced muscle regeneration of neonatal chimeras is shown in Fig. 4. The GFP+ muscle fibres of a neonatal bone marrow chimera (Fig. 4B) or a fetal liver cell chimera (Fig. 4D, F) could be detected. The frequencies of GFP+ fibres are summarized in Table 1. It is evident that the frequencies of GFP+ fibres in radiation-chimeras do not exceed 2%. They show better reconstitution efficiency in their lymphocyte chimerisms than neonatal chimeras. It is interesting to note that the donor bone marrow or fetal liver cell number of the neonatal chimeras was only 1:4-1:40 (for bone marrow cells) or 1:200-1:400 (for fetal liver cells) of adult radiation-chimera experiments; however, the frequencies of GFP+ fibres in mice with lower lymphoid chimerisms are similar to those of radiation chimeras. GFP+ fibres were also observed in the CTX-untreated neonatal chimeras (mdx as recipients) (Table 1; Experiment 6 and 7). This result suggested that the donor-
Muscular regeneration in neonatal chimeras derived bone marrow (Experiment 6) or fetal liver (Experiment 7) cells participate in the normal muscular regeneration in mdx mice. Muscle reconstituting efficiencies of adult bone marrow and fetal liver cells were compared. As summarized in Fig. 5, when the efficiencies were calculated as GFP* fibres/injected cell numbers, fetal liver gave better results than bone marrow.
cells. Fetal livers may contain a higher frequency of muscle progenitor cells than bone marrow.

**GFP+ mononuclear cells in the intact muscles of neonatal chimeras**

During the course of the study, we observed several GFP+ fibres in the CTX-untreated muscle of neonatal bone marrow (Fig. 4H) (C57BL/6 as recipient) as well as fetal liver cell chimeras (data not shown), but the frequency was low. It is evident from the H-E stained picture that the GFP+ fibre has a peripheral nucleus, suggesting that the fibre was generated without damage by CTX. We speculate that these fibres were derived from GFP+ satellite cells in the normal muscle. In accordance with this observation, we detected GFP+ mononuclear cells residing beneath the laminin-positive basal lamina of another CTX-untreated TA muscle (Fig. 4I). This is the typical position in which muscle satellite cells reside. To investigate which of the cells are myogenic progenitor cells, possibly satellite cells, and whether they contributed to muscle generation, we performed a muscle explantation study. After 4 days of explantation culture, desmin+ and GFP+ mononuclear cells were found attached to a single muscle fibre (Fig. 4J-L, M-O).

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**Table 1. Chimerisms of lymphocytes and muscle fibres**

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<th>Treatment</th>
<th>Total fibre (2)</th>
<th>GFP+ fibre</th>
<th>% reconstitution (3)</th>
<th>% (spleen) chimerism (4)</th>
<th>Weeks after reconstitution (5)</th>
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(1) Left VL (Experiment 1), right TA (Experiments 2-6) or left (L) or right (R) TA (Experiment 7) muscles were examined.

(2) Total fibre numbers were counted.

(3) GFP+ fibre/total fibre was calculated.

(4) % chimerism in the spleen (GFP+ lymphocytes/total splenic lymphocytes) were calculated.

(5) Mice were killed and examined at the indicated weeks after the reconstitution.

(6) Left VL (Experiment 1) or right TA (Experiments 2-5) muscles were treated with cardiotoxin (CTX) and muscles were examined.

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**Fig. 5.** Muscle-reconstituting efficiencies of bone marrow and fetal liver cells. Muscle-reconstituting efficiencies of neonatal bone marrow and fetal liver cells were compared as a factor of GFP+ muscle fibres/injected cell numbers. From the data shown in Table 1, both CTX-treated normal C57BL/6 and CTX-untreated mdx recipient mice were calculated.
suspension from CTX-untreated VL muscles was obtained and transplanted into CTX-treated TA muscles of mdx/scid mice. As shown in Fig. 4R and T, muscles of the second recipients showed GFP+ fibres that were derived from intact muscles of neonatal chimeras. Collectively, these results suggest that the GFP+ cells are located in the muscle as possible satellite cells and may be participating in muscular generation upon muscle damage, as well as under physiological condition.

Discussion

The chicken beta-actin promoter and cytomegarovirus enhancer-driven EGFP gene transgenic mouse strain is a powerful tool for studies tracing cell migration and differentiation in animals. The muscles of these mice express ubiquitous and strong fluorescence (Okabe et al., 1997), and this prompted us to apply these mice to bone marrow transplantation and muscle regeneration experiments. In contrast to earlier bone marrow transplantation studies that used nuclear β-galactosidase, the Y chromosome (Ferrari et al., 1998; Gussoni et al., 1999; Bittner et al., 1999) or GFP with the aid of anti-GFP antibody (Orlic et al., 2001) as markers for the detection of donor-derived skeletal or cardiac muscle fibres, GFP fluorescence can easily be detected by flow cytometry or fluorescence microscopy (Chalfie et al., 1994; Kawakami et al., 1999a) without any cofactor for light emission or any specific staining procedures. As shown in this report, although the frequency was not high enough, GFP+ fibres could be detected in the muscles of chimeric mice.

It has long been known that the neonatal injection of allogeneic cells induces tolerance to alloantigens of the donor cells and frequently establishes a chimeric state. Very recently, Liechty et al. (Liechty et al., 2000) described the transplantation of human mesenchymal stem cells in utero into sheep at an early gestational period and the site-specific differentiation of these cells into a variety of tissues, such as chondrocytes, adipocytes, myocytes, cardiomyocytes, and thymic and bone marrow stromas, without any detectable immune response against the human cells. Butler et al. (Butler et al., 2000) showed that neonatally induced tolerance to alloantigens in rats persisted, and skeletal tissue allografts survived, even though the rate of blood cell chimerism was around 3%. Thus transplantation to neonatal or embryonic animals is a promising approach for stem cell therapy for hereditary diseases in animal models. The origin of satellite cells and the time at which they first migrate into muscle tissues are not yet clear. Cosu et al. (Cosu et al., 1983) reported that satellite cells have a different sensitivity to phorbol ester than myoblasts. Taking advantage of this characteristic, the authors showed that satellite cells appear between day 16 and 18 of embryonic development in mice. Muscle satellite cells account for about 30% of sublaminar muscle nuclei in neonatal mice, and this level decreases to less than 5% in adult mice (for reviews, see Campion, 1984; Mazanet and Franzini-Armstrong, 1986; Cosu and Molinaro, 1987; Bischoff, 1994).

These observations strongly suggested to us the idea of introducing muscle precursor cells at the earliest possible time, for example at birth. In the present study, neonatal injection of allogeneic bone marrow or fetal liver cells combined with busulfan treatment successfully induced a chimeric state in both muscular precursor cells and hematopoietic cells. Neonatal mice make it necessary to limit the cell dose and volume of intravenous or intrahepatic injections; however, neonatal chimeras produced similar muscular reconstitution to adult radiation chimeras, although neonates have less efficient blood chimerisms. The immune response against proteins to viral vectors or to newly introduced gene products presents a new obstacle for both gene therapy and cell therapy. Once the mice achieve radiation-induced or neonatally induced immunological tolerance, they can be challenged by another injection of cell transplantation without any immune response.

In order to examine whether donor cells migrate into muscles, we searched for GFP+ cells in the CTX-untreated muscles of neonatal chimeras. As shown in Fig. 4H,I, GFP+ fibre (H) as well as the GFP signal beneath the laminin-positive layer (I) was present. We then explanted muscle fibres and cultured them for 4 days in vitro under non-differentiating conditions. It was reported that naïve desmin+ single human muscle satellite cells in culture develop into two types of cells, one fuses into myotubes and the other persists for weeks among the myotubes (Baroffio et al., 1996). The former expresses alpha sarcomeric actin, whereas the latter expresses desmin. It is well documented that quiescent satellite cells in the muscle express neither muscle-specific markers, such as desmin and myosin, nor the MyoD family of muscle-specific regulatory molecules. However, after muscle injury (Helliwell, 1988; Saito and Nonaka, 1994; Rantanen et al., 1995; Molnar et al., 1996) or short term culture in vitro (Allen et al., 1991; Kaufman et al., 1991; Creuzet et al., 1998), satellite cells became desmin+ and MyoD+. This suggests that the desmin+ cell that attaches to a single fibre is probably a muscle satellite cell. Such cells were observed in the muscle fibre cultures in our present study (Fig. 4L,O), and they are GFP+. GFP+ donor bone marrow or fetal liver cells may be recruited as muscle satellite cells and may participate in muscle regeneration in mdx or CTX-treated muscle.

We then examined whether the fibre-attached cells express M-cadherin (Donalies et al., 1991; Irintchev et al., 1994; Bornemann and Schmalbruch, 1994; Beauchamp et al., 2000), but they were negative in our experiments (data not shown). Beauchamp et al. (Beauchamp et al., 2000) reported that some (~20%) fibre-attached satellite cells in vitro do not express M-cadherin. We observed a few fibre-attached cells, so it is possible that M-cadherin-negative fibre-attached satellite cells were detected in these earlier experiments. To examine whether the GFP+ mononuclear cells become GFP+ fibres, we transplanted the single cell suspension from muscles of neonatal chimeras into immunodeficient mdx/scid secondary recipients. As shown in Fig. 4R,T, GFP+ fibres were generated. Muscle is a highly vascularised tissue and therefore contamination from circulating blood cells can not be excluded; however, the results suggest that the GFP+ cells residing in the CTX-untreated muscles are muscle precursor cells, probably satellite cells.

Recently, De Angelis et al. (De Angelis et al., 1999) reported that the large majority of mouse clones showing typical satellite cell morphology were derived from the dorsal aorta not from somites. Since the cells express several vascular cell markers, the postnatal satellite cells may be derived from a vascular lineage. In the fetal liver, aorta-gonad-mesonephros (AGM)-area-derived hemangioblasts differentiate to both endothelial cells for liver vessels and hematopoietic stem cells.
(Miyajima et al., 2000). Endothelial precursors also arise from the AGM (Ohneda et al., 1998). Because the AGM area appears to be attached in clusters to the dorsal aorta, the precursors of muscle satellite cells, vascular endothelial cells and hematopoietic cells may derive from the same origin at an early embryonic stage. They may move to fetal liver and then to bone marrow in adults. Thus, the bone marrow and fetal liver may have similar cell populations from which various types of cells arise. As shown in Fig. 5, fetal liver cells exceeded bone marrow cells in their myogenic potentials, and it suggests that fetal livers contained higher proportion of myogenic progenitors than adult bone marrows.

The frequency of donor-derived muscle fibres is still too low for the treatment of muscular dystrophies. There are several approaches to overcome these problems. First, it is necessary to enrich the muscular precursor cells from bone marrow or fetal liver cells by using various monoclonal antibodies against cell-type-specific surface markers, such as c-Met, VEGF-receptor, Sca-1 and so on. Further trials to search for novel markers are also necessary. Second, it is not yet known what kind of adhesion molecules are required for the migration of precursor cells into muscle. Neonatal or radiation-induced tolerance, combined with injections of various monoclonal antibodies against adhesion molecules, will answer this question. Lastly, developing or regenerating muscle may produce certain kinds of chemokines or cytokines that control the trafficking of precursor cells. These mediators and their receptors are likely to be critical for their migration. HGF and TGFβ are possible chemotactic factors for adult muscle satellite cells of the rat (Bischoff, 1997), but it is not yet known what kind of factors are responsible for bone marrow- or fetal liver-derived precursors. Although the establishment of tolerance may not be immediately applicable to clinical trials, the experimental system, together with the above-mentioned strategies for the proper reconstitution of muscle, will provide valuable information for future clinical application.

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References


Muscular regeneration in neonatal chimeras


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