Original Article

Role of Transplanted Bone Marrow Cells in Response to Skeletal Muscle Injury

(bone marrow cells / skeletal muscle regeneration / skeletal muscle injury / transplantation / irradiation)

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Abstract. The recently discovered capacity of bone marrow cells (BMCs) to contribute to injury-induced skeletal muscle regeneration has brought new possibilities in the treatment of skeletal muscle diseases. However, a suitable method of BMC transplantation usable for such therapy has to be established. In this work, recipient mice were intramuscularly injected with cardiotoxin, then whole-body lethally irradiated to eradicate satellite cells in their injured tibialis anterior (TA) muscles and to suppress haematopoiesis, and subsequently intravenously transplanted with *lacZ*$^+$ BMCs with the aim to investigate the role of exogenous BMCs in response to skeletal muscle injury. Seven to 33 days after grafting, recipient TA muscles were examined to detect donor-derived X-gal$^+$ cells and analysed by quantitative PCR. In injured recipients' muscles, X-gal positivity was identified 14 and 33 days after grafting in some infiltrating neutrophils and macrophages, infrequently in fibroblasts of endomysium, and in many large multinucleated cells (devoid of myogenic markers desmin and nestin) resembling foreign body giant cells situated in the vicinity of necrotic muscle fibres. qPCR confirmed the presence of transplanted *lacZ*$^+$ BMCs in injured recipients' muscles. Our results proved the ability of intravenously transplanted adult BMCs to settle in injured muscles and generate blood cells that infiltrated endomysium and took part in the cleaning reaction. After inhibition of endogenous myogenesis, BMCs were not able to participate in formation of new muscle fibres due to persisting necrosis of degenerated muscle fibres. Instead, BMCs attempted to resorb necrotic structures, which confirmed the indispensable role of bone marrow-derived macrophages in skeletal muscle regeneration.

Introduction

The bone marrow (BM) represents a unique heterogeneous cell population comprising different cell types at various stages of maturity including haematopoietic stem cells and their progeny (such as lymphoid progenitors differentiating into lymphocytes and myeloid progenitors giving rise to granulocytes, monocytes, erythrocytes and megakaryocytes), mesenchymal (stromal) stem cells, multipotent adult progenitor cells, and endothelial progenitors. Recent studies have shown that cells derived from BM could participate in the regeneration process of non-haematopoietic tissues, e.g. of the skeletal muscle (Ferrari et al., 1998; Gussoni et al., 1999; Fukada et al., 2002; Camargo et al., 2003; Corbel et al., 2003; Doyonnas et al., 2004; Abedi et al., 2005, 2007; Dezawa et al., 2005; Sacco et al., 2005; Luth et al., 2008), liver (Lagasse et al., 2000; Camargo et al., 2004; Willenbring et al., 2004) or neural tissue (Brazelton et al., 2000; Mezey et al., 2000; Priller et al., 2001). In the case of the skeletal muscle, haematopoietic stem cells (HSCs) take part in the regeneration more probably than mesenchymal stem cells (Gussoni et al., 1999; Camargo et al., 2003; Corbel et al., 2003; Doyonnas et al., 2004; Sacco et al., 2005; Abedi et al., 2007). Camargo et al. (2003) and Corbel et al. (2003) reported that even a single haematopoietic stem cell contributed to regeneration of the skeletal muscle after its transplantation to mice with muscle injury. Further investigation has revealed that one type of HSC derivatives, the myelo-

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Abbreviations: BM – bone marrow, BMCs – bone marrow cells, GMA – glycolmethacrylate, HSCs – haematopoietic stem cells, qPCR – quantitative polymerase chain reaction, TA – tibialis anterior.

monocytic cells, is the most important intermediary in the phenomenon of HSC contribution to the skeletal muscle regeneration (Doyonnas et al., 2004; Sacco et al., 2005).

The mechanism of participation of BM-originating cells in the skeletal muscle regeneration has been a subject of recent research, not only because the frequency of BM-derived muscle fibres or their nuclei has still remained very low in conducted experiments (Brazelton et al., 2003; Corbel et al., 2003; Abedi et al., 2005; Palermo et al., 2005). Bone marrow cells (BMCs) could settle in the skeletal muscle and replenish the population of resident muscle stem cells (satellite cells) that differentiate into myoblasts, which then fuse to form myotubes, later on maturing to muscle fibres (LaBarge and Blau, 2002; Luth et al., 2008), or could directly fuse with the existing muscle fibres (Camargo et al., 2003; Doyonnas et al., 2004). It has not been excluded that both these mechanisms may co-exist. In many studies, contribution of BM-derived cells to the skeletal muscle regeneration was documented after whole-body irradiation of recipient mice, transplantation of BMCs and toxin-induced injury of the host skeletal muscle (Ferrari et al., 1998; Camargo et al., 2003; Corbel et al., 2003; Abedi et al., 2005, 2007; Luth et al., 2008). Noteworthy, Palermo et al. (2005) reported that BMCs contributed to the skeletal muscle regeneration without the abovementioned experimental interventions and that this phenomenon could occur in response to physiological stress.

Transplantation of HSCs and their progenitors has been used in human medicine for many years in the treatment of some severe haematological diseases and therefore, methods of BM isolation and its processing for the clinical use have been well elaborated. Promising results of recent research and the clinical use of HSCs raise hope of patients with skeletal muscle diseases in their stem cell-based therapy. In many transplantation experiments describing BMC contribution to the skeletal muscle regeneration, recipient mice were whole-body irradiated with a lethal or sublethal dose and within the next 24 h, a suspension of labelled BM derived cells was transplanted to these animals. After several weeks, during which the recipients had recovered, a toxin injury of the host skeletal muscle was induced (Ferrari et al., 1998; Camargo et al., 2003; Corbel et al., 2003; Abedi et al., 2005, 2007; Luth et al., 2008). In human patients, however, a disease, damage or injury of the skeletal muscle occurs first and the treatment follows. Moreover, the capacity of satellite cells to regenerate the skeletal muscle is severely reduced in most cases.

Therefore, in our study we set the experimental design closer to the situation of human patients and intravenously transplanted mouse *lacZ*$^+$ BMCs into whole-body lethally irradiated recipient mice after their tibialis anterior (TA) muscles had been injured. We tracked the transplanted cells with the aim to investigate their role in the recipient animals, particularly in the injured TA muscles in which satellite cells were eradicated by whole-body lethal irradiation. The injured as well as intact contralateral TA muscles of the transplanted mice were thoroughly morphologically examined and analysed by quantitative polymerase chain reaction (qPCR) for the *lacZ* gene DNA content, 7, 14 and 33 days after the muscle injury, whole-body irradiation and bone marrow transplantation.

Material and Methods

Experimental animals

B6;129S-Gt(ROSA)26Sor (ROSA26) mice and B6129SF2/J (F2 hybrid) mice were purchased from Jackson Laboratories (Bar Harbor, ME). The mice used for the experiments were housed in groups of two in a temperature- and humidity-controlled colony room that was maintained at a 12-h light/dark cycle. Food and water were available ad libitum throughout the experiment. The investigation was approved by the Ethical Committee supervising procedures in experimental animals at the Faculty of Medicine in Hradec Králové, Charles University in Prague, Czech Republic.

Skeletal muscle injury

Muscle injury was provoked in F2 hybrid recipient mice by injecting 75 µl cardiotoxin (Naja mossambica mossambica; 0.06 µg/µl diluted in 0.9% NaCl solution; Sigma-Aldrich, St. Louis, MO) into the right TA muscle. Before the cardiotoxin injection, the recipient mice were anaesthetized by intraperitoneal administration of ketamine (100 mg/kg; Narkamon 5%, Spofa, Prague, Czech Republic) and xylazine (10 mg/kg; Rometar 2%, Spofa). A short section of the skin above the right TA muscle facilitated the accurate injection of cardiotoxin solution along the longitudinal axis of the muscle, deeply from the ankle to the knee of the mouse leg, using a 27-gauge needle and a 1-ml syringe. After the cardiotoxin injection, the cut skin was sutured using Premilene (B/Braun Melsungen AG**,** Melsungen, Germany). All surgical procedures were performed under aseptic conditions.

Whole-body irradiation and bone marrow transplantation

BMCs were obtained from 8- to 10-week-old ROSA26 *lacZ*$^+$ donor mice. Single-cell suspensions were prepared from the marrow contents of the femurs of mice in PBS (Dulbecco's phosphate-buffered saline, Sigma-Aldrich) containing 2% foetal calf serum.

Recipient animals, 8- to 10-week-old F2 hybrid mice, were exposed to 9 Gy of γ radiation from a ^{60}Co source (Chisotron, Chirana, Prague, Czech Republic) at a dose rate of 1.1 Gy/min, 4 h after the muscle injury. Subsequently, 3 h following the irradiation, suspension of 5×10^6 freshly isolated ROSA26 bone marrow cells/mouse were transplanted intravenously via the tail vein to recipients.

Two types of control experiments were conducted. In the first type, the TA muscles of recipient mice (F2 hybrid) were injured with an injection of cardiotoxin and 4 hours afterwards, the recipients were lethally whole-body irradiated. BMC transplantation was omitted. In the other type of the control experiments, the cardiotoxin-induced injury of the recipients' TA muscles was the only intervention; whole-body irradiation and bone marrow transplantation were not performed.

Histology and X-gal histochemistry

In total, 18 recipient mice were sacrificed by CO_2 inhalation and subsequent exsanguination and then perfused with 4% paraformaldehyde in 0.1 M PB (phosphate buffer; pH 7.6) 7 (N = 6), 14 (N = 6) and 33 days (N = 6) after BMC transplantation. Their right injured and left intact TA muscles were excised and small pieces cut off the collected specimens were immersed in 4% paraformaldehyde in 0.1 M PB (pH 7.6) for 1 h at 4 °C. After thorough washing in saline solution, the specimens were incubated in X-gal solution (pH 7.6) for 24 h at 37 °C to detect donor-derived cells expressing ß-galactosidase. Following subsequent washing, the samples were dehydrated and embedded in paraffin or glycol-methacrylate (GMA) resin. Seven-µm-thick serial sections were cut from paraffin blocks using a microtome and attached to the glass slides covered with gelatin. From GMA resin blocks, 1- or 2-µm-thick serial sections were cut using the ultramicrotome Ultratome Nova (LKB, Bromma, Sweden) and attached to the glass silanized slides (Dako, Glostrup, Denmark). Every tenth slide with a paraffin section was stained with nuclear red. Haematoxylin-eosin staining was applied to several slides to allow thorough histological examination. The presence of calcium was determined by von Kossa impregnation and alizarin red staining.

Immunohistochemistry

Immunohistochemical detections were performed by the indirect three-step LSAB method in paraffin-embedded sections following X-gal histochemistry. After deparaffinization and rehydration of sections, original conformations of epitopes were retrieved using microwaves (in sodium citrate solution for 3 × 5 min at 700 watts). Endogenous peroxidase was blocked in 1% H_2O_2 (3 × 10 min) and then the sections were incubated in 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were incubated with primary rabbit monoclonal anti-desmin (clone Y266, Abcam, Cambridge, UK) or mouse monoclonal anti-nestin (clone Rat-401, DSHB, Iowa City, IA) antibody overnight at 4 °C and after washing in PBS, they were exposed to anti-rabbit or anti-mouse secondary biotinylated antibodies (Jackson ImmunoResearch Laboratories) for 45 min at room temperature. After rinsing, sections were incubated with streptavidin conjugated to horseradish peroxidase (Sigma-Aldrich) for 45 min and then the reaction was developed with 3,3-diaminobenzidine tetrahydrochloride (Fluka, Sigma-Aldrich). Sections were dehydrated, counterstained with nuclear red and mounted in DPX. To avoid false immunopositivity, serial sections were processed according to the same protocol, but primary antibodies were omitted. Tissue sections were examined in an Olympus BX51 microscope equipped with a DP71 camera (Olympus Corporation, Tokyo, Japan).

Analysis of bacterial LacZ DNA content by qPCR

For qPCR analysis, in total 18 recipient mice were sacrificed by CO_2 inhalation and subsequent exsanguination 7 (N = 6), 14 (N = 6) and 33 days (N = 6) after BM transplantation. Their right injured and left intact TA muscles were excised, frozen in liquid nitrogen and stored at –80 °C. From three transplanted recipient mice in each group (in total nine mice), BMCs were obtained from the femurs and tibias by flushing with PBS solution, frozen in liquid nitrogen and stored at –80 °C.

DNA was isolated from skeletal muscle and bone marrow samples using DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). Quantitative PCR was performed in triplicate with 20 ng of DNA using the TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and pre-designed gene assays for the bacterial *lacZ* gene (ecoLacZ_Q1; GENERI BIOTECH s.r.o, Hradec Kralove, Czech Republic) and mouse housekeeping *polr2a* gene (mPolr2a_G1; GENERI BIOTECH s.r.o.). Cycling conditions (for both LacZ and Polr2a products) were 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative expression ratio was then calculated from the ΔCt_{target} and $\Delta Ct_{housekeeping}$ values as described previously (Fuksa et al. 2010).

Statistical analysis of qPCR results

All values in the figures were expressed as mean ± SE of the mean. Student's *t*-test (unpaired, two-tailed) was used for comparison between experimental data. A P value < 0.05 was considered as statistically significant.

Results

Histological structure of injured skeletal muscles of irradiated and transplanted mice

Seven days after the muscle injury, whole-body irradiation and BMC transplantation, muscle fibres in sites of the injury were necrotic. Areas with the degenerated muscle fibres were infiltrated by inflammatory cells, mainly by the neutrophils. Nevertheless, the inflammatory infiltrates were not numerous. Von Kossa impregnation and alizarin red staining revealed calcification in the necrotic muscle fibres (Fig. 1A). No X-gal$^+$ cells were identified in the sites of the injury and in the remaining intact parts of the TA muscles as well. Desmin immunoreactivity was only detected in the uninjured muscle fibres and nestin expression was restricted to the areas of neuromuscular and myotendinous junctions of the intact muscle fibres.

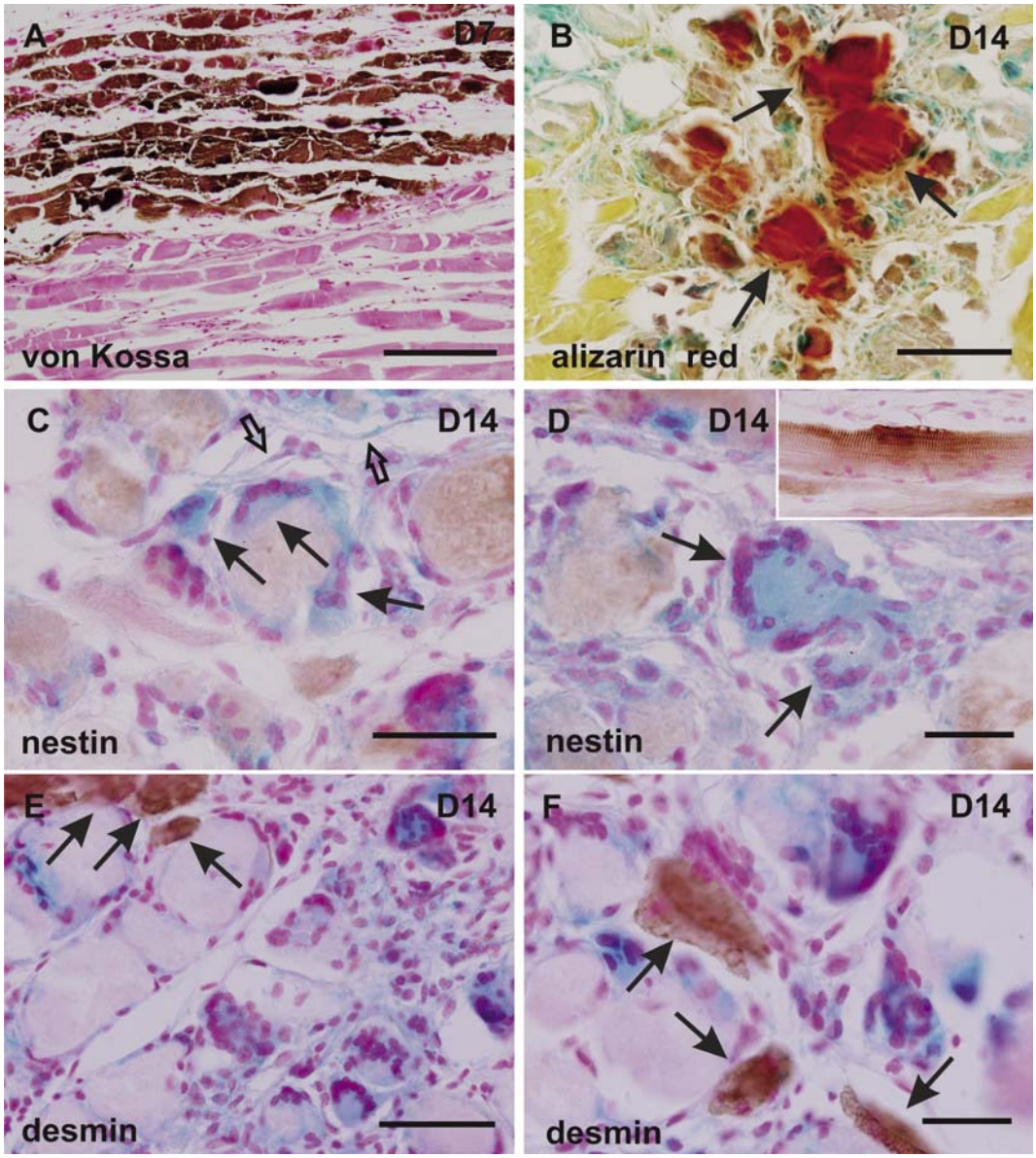

Fig. 1. Histochemical detection of donor-derived *lacZ*$^+$ cells, proof of calcification (A, B) and imunohistochemical detections of nestin (C, D) and desmin (E, F) in the injured TA muscles of the transplanted mice. The necrotic sarcoplasm of the degenerated muscle fibres in the injured muscles, 7 (D7) and 14 days (D14) after the cardiotoxin injection, was calcified (arrows) as determined by von Kossa impregnation (A, calcium salts are dark brown to black) and alizarin red staining (B, calcium is stained red, blue colour of the *lacZ*$^+$ cells changed to green due to the staining). Nestin was not detected in the X-gal$^+$ (blue) large multinucleated cells (C, D, arrows). "Empty" arrows in C depict X-gal$^+$ fibroblasts of endomysium. Insert in D shows nestin expression in the region of a neuromuscular junction of an intact muscle fibre situated in the same section as a positive control for the nestin detection. The X-gal$^+$ large multinucleated cells did not express desmin, whereas surviving muscle fibres revealed strong desmin immunoreactivity (E, F, arrows). Figs. 1A, C, D, E, F are counterstained with nuclear red; scale bars A 400 µm, B 200 µm, C 100 µm, D, E, F 50 µm.

The necrotic calcified sarcoplasm of the degenerated muscle fibres persisted and was observed in the injured TA muscles excised from the transplanted mice 14 days after the muscle damage (Fig. 1B). In the vicinity of the degenerated muscle fibres in the sites of the injury, large multinucleated X-gal$^+$ cells were identified (Fig. 2A-C). The majority of them were in close contact with the necrotic muscle fibres, and interestingly, some of these cells even seemed to envelope the necrotic sarcoplasm (Fig. 2B-C). These X-gal$^+$ cells were of irregular shape and their oval or irregularly shaped nuclei were not arranged or occasionally, they were accumulated forming a crescent at the periphery of the cells (Fig. 2B). According to their morphology, the large multinucleated X-gal$^+$ cells resembled foreign body giant cells that were formed by fusion of activated macrophages and indicated a chronic inflammation. Infrequently, X-gal$^-$ large multinucleated irregularly shaped cells were present in the same location as the X-gal$^+$ cells. X-gal positivity was also identified in several cells of endomysium, the

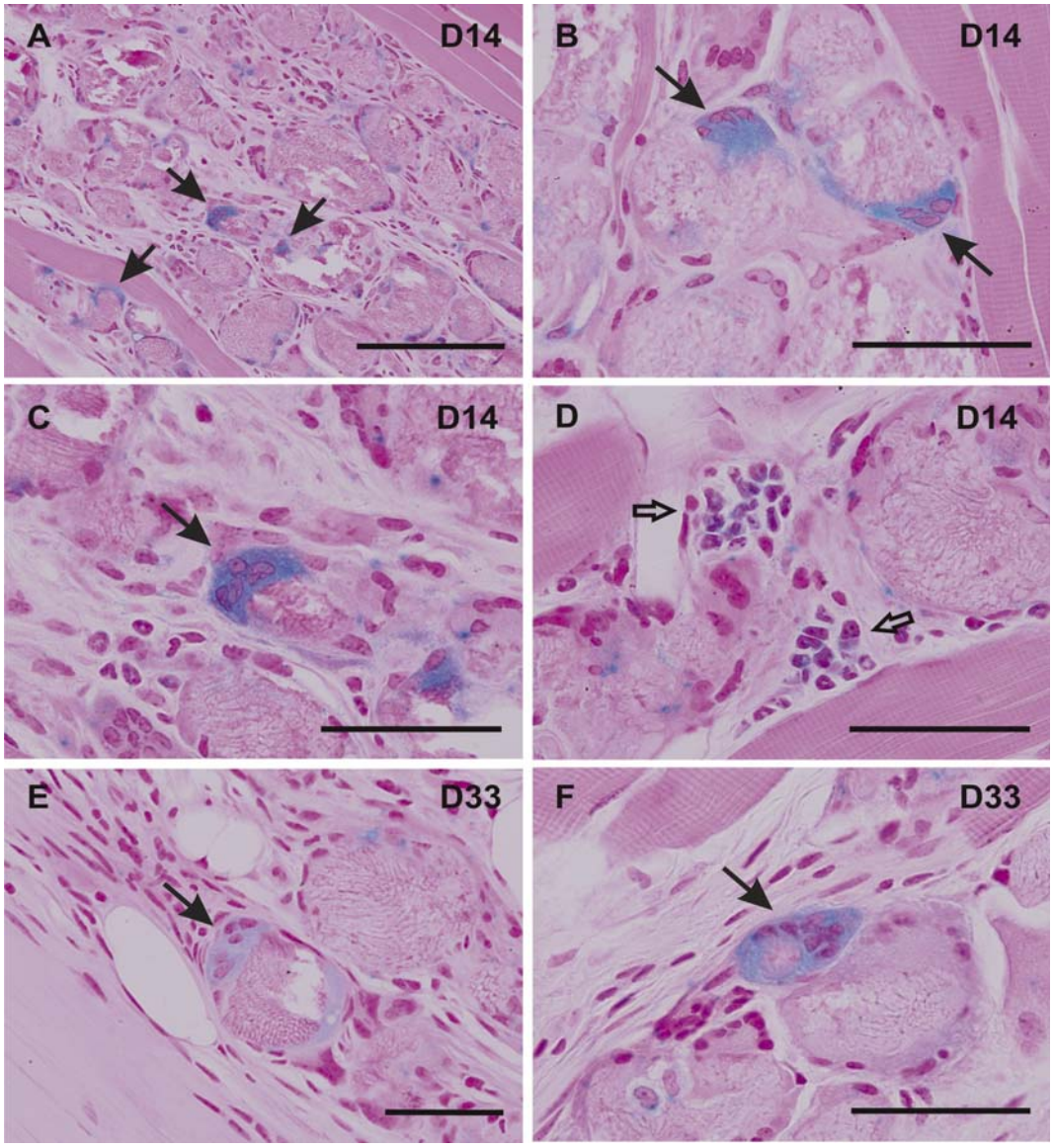

Fig. 2. Histological structure of the injured TA muscles and histochemical detection of donor-derived *lacZ*$^+$ cells 14 days (A, B, C, D) and 33 days (E, F) after BMC transplantation. X-gal$^+$ large multinucleated irregularly shaped cells were present in sites of the injury 14 days (D14) as well as 33 days (D33) following the transplantation (arrows). They were situated in the vicinity of the necrotic muscle fibres; however, they did not have a typical morphology of myotubes. They resembled foreign body giant cells formed by fusion of macrophages. X-gal$^+$ cells of inflammatory infiltrates were found in endomysium (D, "empty" arrows). GMA 1–2 µm thick sections; nuclei of cells are darkly counterstained with nuclear red; scale bars A 200 µm, B 100 µm, C, D 50 µm, E, F 100 µm.

connective tissue of the injured muscles. Spindle-shaped cells with an elongated or oval nucleus and thin projections might represent X-gal$^+$ fibroblasts; nevertheless, their findings were sporadic (Fig. 1C). By contrast, X-gal$^+$ cells of inflammatory infiltrate were more numerous. In the surroundings of the degenerated muscle fibres, groups of X-gal$^+$ neutrophils were still present (Fig. 2D) and many X-gal$^+$ macrophages occurred. In the TA muscles 14 days after the injury, nestin was not expressed in any type of X-gal$^+$ cells, including the large multinucleated irregularly shaped cells (Fig. 1C-D). In sites of the injury, muscle fibres revealing normal morphology expressed desmin, as distinct from the degenerated muscle fibres (Fig. 1E-F).

Thirty-three days after the cardiotoxin injection, the sites of the muscle injury were constituted by necrotic calcified degenerated muscle fibres surrounded with X-gal$^+$ large multinucleated irregularly shaped cells (Fig. 2E-F) and endomysium with rare X-gal$^+$ fibroblasts and macrophages. In comparison with the histological structure of the injured TA muscles examined 14 days after the muscle damage, the number of the X-gal$^+$ large multinucleated cells as well as the inflammatory infiltrate cells slightly decreased; on the contrary, the amount of the muscle connective tissue including fibroblasts correspondingly increased. Immunohistochemical detection of nestin confirmed that the X-gal$^+$ large multinucleated irregularly shaped cells did not ex-

press this intermediate filament protein. Desmin immunohistochemistry allowed visualization of viable muscle fibres situated between the degenerated muscle fibres in the sites of the injury.

In all transplanted mice, the histological structure of the left intact TA muscles was found normal and no X-gal$^+$ cells were noticed.

Histological structure of the injured skeletal muscles of irradiated and non-transplanted mice

In the control mice, lethally whole-body irradiated 4 h after the cardiotoxin injection, the injured TA muscles were examined 7 and 14 days after the muscle damage. Longer survival periods were not studied to prevent the mice from suffering from symptoms of the lethal irradiation and their upcoming death. Seven as well as 14 days after the cardiotoxin injection, in the sites of the injury, the degenerated muscle fibres were necrotic and the cells of inflammatory infiltrate occurred in the endomysium. No X-gal$^+$ cells or signs of regeneration were observed (Fig. 3A).

Histological structure of the injured skeletal muscles of non-irradiated and non-transplanted mice

In the control mice with the injured TA muscles (without whole-body irradiation and BMC transplantation), the damaged muscles fully regenerated. Seven days after the cardiotoxin injection, newly formed myoblasts, myotubes and muscle fibres with centrally located nuclei were noticed in the sites of the injury (Fig. 3B). After the next seven days, the nuclei of the newly developed muscle fibres became peripherally situated and 33 days after the injury, the sites of the damage were hardly recognizable because regeneration of the muscles had been completed. No X-gal$^+$ cells could be indentified in these control muscles.

Analysis of bacterial lacZ DNA content in the TA muscles and the bone marrow of transplanted mice

Analysis of the bacterial *lacZ* gene DNA content by the qPCR method confirmed that donor-derived cells, or their nuclei, were present in the injured TA muscles of recipients at all time periods, i.e. 7, 14 and 33 days after BM transplantation, as shown in Fig. 4. A progressive rise in the donor cell DNA content in the injured TA muscles during time was observed. The highest mean value reached 23 % of the *lacZ* DNA content in the TA muscles of the donor ROSA26 mice, in the injured TA muscles examined 33 days after the transplantation. There was a statistically important rise between the 7th

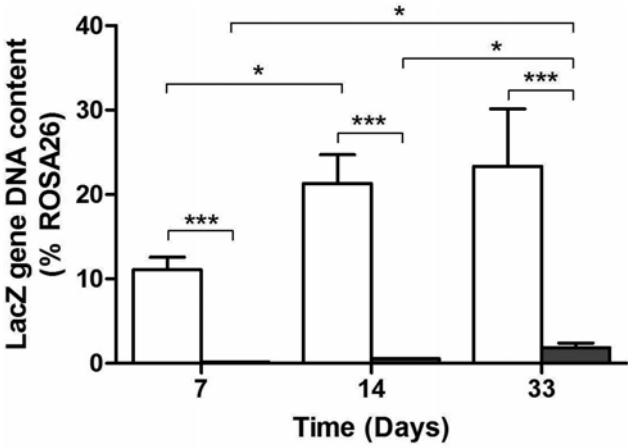

Fig. 4. Analysis of bacterial *lacZ* DNA content in the injured TA muscles (white columns) and the intact contralateral TA muscles (grey columns) of the transplanted mice by the qPCR method, 7, 14 and 33 days after the cardiotoxin injection. Values are means ± SE. All values in the graph were statistically importantly different ($P < 0.001$) from the value of the *lacZ* DNA content in the intact TA muscles of donor ROSA26 mice which represented 100 %. * $P < 0.05$; *** $P < 0.001$

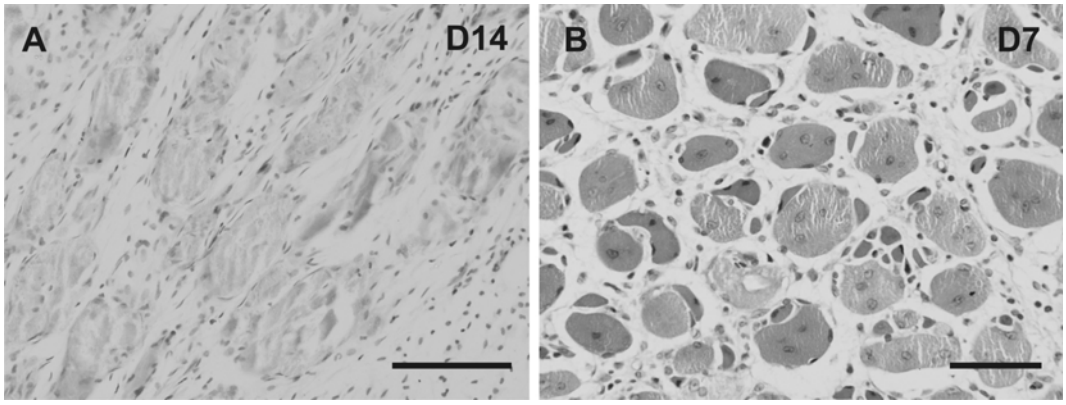

Fig. 3. Histological structure of the injured TA muscles and X-gal histochemical detection in control mice. In the control mice, whole-body irradiated 4 h after the muscle injury, with omission of BMC transplantation, no X-gal$^+$ cells or signs of muscle regeneration were observed (A). In the other type of control experiment, when the control mice were only injected with cardiotoxin (and non-irradiated), new myoblasts, myotubes and muscle fibres with centrally located nuclei were formed in the sites of the injury, 7 days after the muscle damage (B). Fig. A is counterstained with nuclear red, Fig. B is counterstained with haematoxylin-eosin; scale bars A 200 μm, B 50 μm

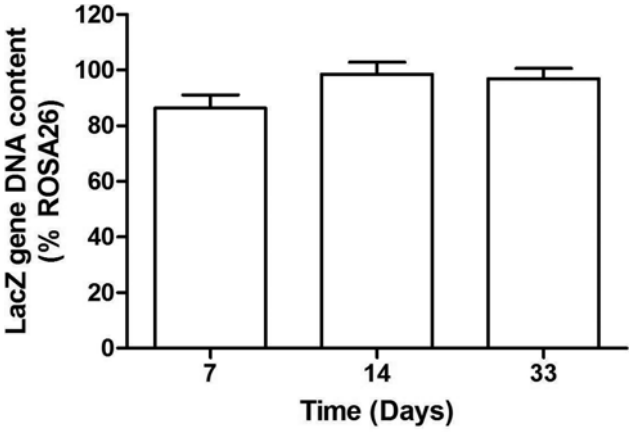

Fig. 5. Analysis of bacterial *lacZ* DNA content in the bone marrow of transplanted mice by the qPCR method, 7, 14 and 33 days after the muscle injury. Values are means ± SE. The bacterial *lacZ* DNA content in the bone marrows of donor ROSA26 mice represented 100 %. Statistical analysis was not done because of the low number of mice in each group (N = 3).

and 14th day following the transplantation ($P < 0.05$) in the injured TA muscles of the recipients. Interestingly, in the intact contralateral TA muscles of the recipient mice, the *lacZ* DNA content rose during time as well, statistically importantly between the 7th and 33rd day and the 14th and 33rd day after the transplantation ($P < 0.05$). Altogether, the donor cell DNA content was manifold higher in the injured than in the intact TA muscles of the recipients. A statistically important difference was found between the injured and the intact muscles at all time periods, i.e. 7, 14 and 33 days after the transplantation ($P < 0.001$).

The bone marrow of three transplanted recipient mice from each group was analysed for the presence of the *lacZ* gene by the qPCR method. The results, shown in Fig. 5, confirmed that donor-derived BMCs settled in the recipients following the transplantation and replaced the endogenous BMCs destroyed by the lethal whole-body irradiation. The statistical analysis of these results was not done because of the low number of mice in each group. Nevertheless, it was apparent that the values of the donor cell DNA content got closer to 100 % of the *lacZ* gene DNA content in the BM of the donor ROSA26 mouse, which confirmed the efficacy of BMC transplantations in our experiments.

Discussion

In this work, recipient mice were lethally whole-body irradiated 4 h after the cardiotoxin-induced injury of their TA muscles and subsequently, 3 h following the irradiation, BMCs freshly isolated from *lacZ*-expressing donor ROSA26 mice were intravenously transplanted to recipients. The aim of these experiments was to study the role of the transplanted BMCs in response to the injury of the recipient skeletal muscles.

Injection of cardiotoxin into the skeletal muscle induces degeneration of the muscle fibres, but does not affect satellite cells, blood vessels and muscle innervation (Couteaux et al., 1988). Necrosis of the muscle fibres caused by the toxin occurs very quickly, as early as 30 min after the injection (Ownby et al., 1993). Necrosis triggers local acute immune reaction and inflammatory cells immediately infiltrate the site of the injury. The necrotic degenerated muscle fibres are removed by macrophages that also participate in the activation of the satellite cells by secreting growth factors (Lescaudron et al., 1999). The activated satellite cells proliferate, differentiate into myoblasts that fuse during the third day after the muscle damage to form myotubes, finally maturing to muscle fibres (Couteaux et al., 1988). In our experiments, the recipient mice were lethally whole-body irradiated very early, 4 h after the cardiotoxin injection, to inhibit the quick spontaneous regeneration of the injured muscles, and thus to imitate the situation in human patients with severely reduced ability of skeletal muscle regeneration. We supposed that the irradiation affected the activated or proliferating satellite cells and caused their cell death. The absence of signs of regeneration in the injured TA muscles of the control mice that were lethally whole-body irradiated 4 h after the cardiotoxin injection and the findings of full regeneration in the injured TA muscle of the control mice that were only injected with cardiotoxin supported our assumption. The lethal whole-body irradiation also suppressed haematopoiesis. In our experiments, the BMC transplantations were successful and a high degree of chimerism was achieved as confirmed by the analysis of the donor cell DNA content in the BM of recipient mice determined by the qPCR method.

X-gal positivity representing the donor cell-derived origin was identified in the injured recipients' muscles in the large multinucleated irregularly shaped cells situated in the close vicinity of the necrotic degenerated muscle fibres, in infiltrating neutrophils and macrophages, and infrequently in fibroblasts of the endomysium, 14 and 33 days after the muscle injury. These results of histochemical analysis were in accordance with the results of the *lacZ* DNA content analysis by qPCR. Seven days after the cardiotoxin injection, no X-gal$^+$ cells were observed in the injured muscles, but the mean value of the donor cell DNA content at this time point reached 11.1 % of the *lacZ* DNA content in the TA muscle of the donor ROSA26 mouse. A similar discrepancy arose between the results of histochemical detection of X-gal$^+$ cells and of analysis of donor cell DNA content determined by the qPCR method in the intact contralateral TA muscles of the recipients. Analysis of the *lacZ* gene DNA content accomplished by qPCR represents a very accurate and sensitive method of proving the donor-derived *lacZ* expressing cell occurrence in the tissues of the recipients. However, this method does not enable determination of phenotypes of *lacZ*-expressing cells. On the contrary, histochemistry allows studying the morphology and tissue distribution of the X-gal$^+$ cell types. The disadvan-

tage of this method is that X-gal positivity is only detected in the cells that contain an active form of the enzyme ß-galactosidase encoded by the *lacZ* gene. Cells of the donor origin that do not synthesize or form active β-galactosidase can occur in the recipient tissues and are not detected by X-gal histochemistry. This fact is, according to our opinion, an explanation for the discrepancy between the results of analysis of the *lacZ* gene determined by qPCR and of X-gal histochemical detections. Nevertheless, simultaneous application of these two methods was found advantageous in our work, because they complemented each other.

Fourteen and 33 days after the muscle injury, whole-body irradiation and BMC transplantation, the X-gal$^+$ large multinucleated irregularly shaped cells were identified in the close vicinity of the necrotic degenerated muscle fibres in the sites of the TA muscle injury. These cells did not express desmin, a reliable muscle marker. Desmin represents the main component of intermediate filaments in the adult skeletal muscle and is expressed in all stages of muscle cells during their development (Bignami and Dahl, 1984; Fürst et al., 1989; Sejersen and Lendahl, 1993; Li et al., 1997) as well as in the course of the skeletal muscle regeneration (Vaittinen et al., 2001; Čížková et al., 2009a). Nestin, a unique intermediate filament protein, is generally considered to be a marker of stem/progenitor cells. Nevertheless, it is not expressed in all types of stem/progenitor cells, e.g. it is absent in haematopoietic or embryonic stem cells, and is detected in some immature and rarely, in several mature cells. This protein is usually down-regulated during cell differentiation in the course of development as well as tissue renewal or regeneration and apparently replaced with another type of intermediate filaments that is expressed in mature cells (Mokrý et al., 2004). In the skeletal muscle, nestin was detected at the beginning of development – in myotomal cells in mouse somites (Kachinski et al., 1994). During development and regeneration, nestin copolymerizes with another intermediate filament vimentin that disappears during further differentiation in myoblasts and co-assembles with desmin in maturing myotubes. In mature skeletal muscle fibres, nestin is only expressed in the sarcoplasm of neuromuscular and myotendinous junctions (Carlsson et al., 1999; Vaittinen et al., 1999; Čížková et al., 2009b). In addition to desmin, the X-gal$^+$ large multinucleated cells did not express nestin as well. Therefore, these X-gal$^+$ cells did not belong to myogenic lineage, although they revealed some features of newly formed muscle fibres, such as multiple nuclei and location in the close vicinity of the necrotic sarcoplasm of the degenerated muscle fibres. According to their structure, the X-gal$^+$ large multinucleated cells resembled foreign body giant cells that were formed by fusion of activated macrophages. One to two days after the onset of acute inflammation, circulating monocytes migrate to the site of injury and after their emigration to connective tissue, they undergo transformation into macrophages with a greater capacity for phagocytosis. Macrophages become activated by microorganisms, dead cells, etc., and then secrete various biologically active products, which is typical of chronic inflammation. If a causal agent is a relatively inert foreign body, so-called foreign body granulomas develop. Accumulation of activated macrophages and their fusion into foreign body giant cells induced by interferon γ are characteristic for this distinctive type of chronic inflammation.

Zhao et al. (2009) evaluated cardiotoxin-induced skeletal muscle injury as a suitable animal model for dystrophic calcification. They described the occurrence of large multinucleated cells resembling osteoclasts that were resorbing calcified necrotic degenerated muscle fibres. In the injured TA muscle of transplanted recipient mice in our experiments, calcification of the necrotic sarcoplasm of the degenerated muscle fibres was confirmed by alizarin red staining and von Kossa impregnation. Therefore, the X-gal$^+$ large multinucleated cells situated in the close surroundings of the calcified necrotic muscle fibres could represent osteoclast-like cells. Similarly to the foreign body giant cells, the osteoclasts are able to phagocytose and originate from fusion of activated macrophages. We are aware that osteoclasts could possess endogenous mammalian β-galactosidase activity and thus reveal false X-gal positivity (Coates et al., 2001; Kopp et al., 2007). Nevertheless, we have unambiguous proof that in our experiments, the X-gal$^+$ large multinucleated cells were derived from the transplanted BMCs. Firstly, analysis of the *lacZ* gene DNA content determined by qPCR confirmed that the donor cell-derived nuclei were present in the injured TA muscle of the recipient mice, in the number corresponding to the amount of the X-gal$^+$ large multinucleated cells. Secondly, in the control mice lethally whole-body irradiated after the cardiotoxin injection but not transplanted, no X-gal$^+$ cells were identified in the sites of the muscle injury. Therefore, the X-gal$^+$ cells occurred in the sites of cardiotoxin injection in the transplanted recipients originated in the donor cells. Whether the X-gal$^+$ large multinucleated cells observed in our experiments resembled more foreign body giant cells or osteoclasts, the main question was why they did not resorb the necrotic muscle fibres. In our view, the persistence of the necrotic muscle fibres could be caused by the insufficient number of cells capable of phagocytosis relative to the extent of necrosis and/or by a change of the necrotic tissue characteristics induced by the irradiation that made the necrotic muscle fibres resistant to resorption.

After an intramuscular injection of cardiotoxin, the sites of the injury are infiltrated by macrophages and other inflammatory cells very early, and necrotic degenerated muscle fibres are removed by two or three days (Couteaux et al., 1988). In our experiments, inflammatory infiltrates were not numerous and the necrotic muscle fibres were present seven days after the muscle injury. It is reasonable to assume that transplanted BMCs have to reconstitute haematopoesis affected by lethal whole-body irradiation first, so they proliferate and differentiate to restore all haematopoietic cells including

differentiated blood cells, which is lifesaving for the irradiated mice. After the haematopoesis had been renewed, the progeny of the transplanted BMCs could participate in repair of the injured skeletal muscle. Therefore, in our experiments, inflammatory infiltration of the sites of muscle damage in the irradiated and transplanted mice was delayed in comparison with the control mice only injected with cardiotoxin.

The bone marrow contains mesenchymal (stromal) stem cells as well. Although they are infrequent, they were described to be able to participate in the skeletal muscle regeneration (Dezawa et al., 2005; de la Garza-Rodea et al., 2011). During development, they are a source of many connective tissue cells, including fibroblasts. Therefore, our sporadic findings of X-gal$^+$ fibroblasts in the sites of the muscle injury in the transplanted mice were not surprising. Activated and proliferating fibroblasts of endomysium could be affected by the whole-body irradiation and their regeneration could then be triggered. According to our results, donor BM-derived mesenchymal stem cells and their progeny participate in the process of endomysium renewal.

Several mechanisms by which transplanted cells potentially participate in injury-induced skeletal muscle regeneration have been described. BM-derived cells could travel through circulation to the sites of muscle injury, where they could give rise to muscle satellite cells that effect the muscle regeneration (LaBarge and Blau, 2002; Luth et al., 2008). Another mechanism involves inflammatory infiltrate cells, above all monocytes, macrophages and neutrophils, and their fusion with developing myotubes during muscle regeneration (Camargo et al., 2003; Doyonnas et al., 2004). A different opinion is that skeletal muscle regeneration is entirely mediated by stem cells resident in muscle tissue, because destruction of muscle resident stem cells with high-dose local irradiation results in a long-term deficit in muscle growth and regeneration (Wakeford et al., 1991; Pagel and Partridge, 1999; Heslop et al., 2000). Our results are in agreement with studies confirming the latter mechanism. In our experiments, BMC transplantation did not prevent degeneration of the muscles in which the satellite cells had been eradicated by lethal whole-body irradiation. The appearance of rare X-gal$^-$ mature muscle fibres in the sites of muscle injury in the transplanted mice could be caused by activation and further proliferation and differentiation of resident satellite cells that had survived the irradiation. However, we do not exclude the first two possible mechanisms of BMC contribution to skeletal muscle regeneration. In our work, the satellite cell niche may be damaged by the irradiation and as a result, natural signals necessary for induction of new satellite cell differentiation and new muscle fibre formation, in which transplanted BMCs might participate, could be lacking. A failure of the first regeneration stage when macrophages resorb the necrotic muscle fibres gives evidence of the indispensable role of macrophages in the initiation of the skeletal muscle regeneration process.

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