Improvement of Contraction Force in Injured Skeletal Muscle after Autologous Mesenchymal Stroma Cell Transplantation Is Accompanied by Slow to Fast Fiber Type Shift

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Keywords

Muscle trauma · Regeneration · Stem cells · Fiber type · Fast myosin heavy chain · Slow myosin heavy chain · Shift · Tissue engineering

Summary

Background: Skeletal muscle trauma leads to severe functional deficits, which cannot be addressed by current treatment options. Previous investigation could show the efficacy of a local transplantation (TX) of mesenchymal stroma cells (MSCs) for the therapy of muscle injury. Underlying mechanisms remain to be elucidated. The aim of the present work was to characterize the fiber composition changes following MSC-TX after open crush injury. **Methods:** 20 male SD rats received an open crush trauma of the left soleus muscle. 2.5×10^6 autologous MSCs were transplanted into the crushed soleus muscle of 10 animals 7 days after trauma (group 1, $n = 10$). Control animals received an injection of saline solution (group 2, $n = 10$). Histologic analysis of fibrosis, fiber type composition, and muscle force measurements were performed 28 days after trauma. **Results:** MSC-TX improved muscle force significantly (fast-twitch, treated: 0.76 (0.51–1.15), untreated: 0.45 (0.32–0.73); $p = 0.01$). Tetanic stimulation resulted in a significant increase of force development (treated: 0.63 (0.4–1.21), untreated: 0.34 (0.16–0.48); $p = 0.04$). Histological analyses showed no differences in the amount of fibrotic tissue (treated

vs. untreated, $p = 0.42$). A shift towards fastMHC-positive fibers was observed following MSC-TX (treated vs. untreated; $p = 0.01$ (mm²) or 0.007 (%)). **Conclusion:** This study demonstrated an effect of locally administered MSCs in the treatment of skeletal muscle injuries on a structural level. For the first time a fiber type shift towards fastMHC following MSC-TX after crush injury could be demonstrated and related to MSC-TX. These results might open the discussion of an alternative mode of action of MSCs in tissue regeneration.

Introduction

The treatment of muscle injuries remains an unsolved problem in trauma and orthopedic surgery and sports medicine [1, 2]. The relevance of muscle deficits and scar tissue formation is widely underestimated. Therapeutic strategies to regenerate skeletal muscle tissue are urgently needed, since currently available methods do not serve that purpose.

The reduction of contractile elements, scar formation, and fatty muscle degeneration are the challenges in regaining functional recovery. Following injury also the arrangement of muscle fibers is altered and contributes to limited muscle function. Thus, structure and function are altered after trauma, and regeneration aims at restoring both aspects.

In earlier experiments, cell-based therapies have demonstrated the regenerative potential of stem cells following systemic transplantation [3, 4]. A clear effect on improved mus-

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cle function after trauma was shown after local transplantation of autologous mesenchymal stem cells (MSCs) [5]. A dose-response relationship between the number of transplanted cells and the functional outcome and the persistence of a fraction of the donor cells at the site of trauma was observed while no sex specific differences were found [6, 7].

Several options are discussed as potential mechanisms of action of stem cells or stem cell-like cells leading to the beneficial effects in muscle regeneration. Fusion events are, due to their small number, regarded as subordinate with respect to muscle regeneration [8]. Our own group tracked MSCs non-invasively in vivo and found transplanted cells mainly in the interstitial compartment [9]. Beside the direct differentiation of stem cells into myofibers, a paracrine effect of the cells to support regeneration is discussed. MSCs produce soluble factors (e.g. vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)) [10]. These factors are biologically active, modify local immunological responses, affect the formation of fibrosis, and promote the differentiation and proliferation of myofibers [11–14]. In particular the IGF family has been shown to be a key factor in muscular regeneration. Gnechchi et al. [15] postulated a paracrine mode of action, which they concluded from decreased fibrosis and apoptosis after local injection of MSC-conditioned medium in myocardial infarction [16]. Skeletal muscle fibers can be basically classified as follows: slow (slow myosin heavy chain, type I, aerobic; slowMHC) fast (fast myosin heavy chain, type II, anaerobic; fastMHC), and slow-fast-mixed fibers [12, 17]. In the rat, the soleus muscle contains predominantly slow myosin heavy chain fibers. The physiological regeneration of skeletal muscle follows three sequential phases: i) inflammation with the presence of macrophages, ii) activation and fusion of satellite cells, and iii) the maturation of newly formed myofibers and the development of fibrosis. According to Merrick et al. [12], growth factors of the IGF family are required for establishing fastMHC-positive myotubes during fiber type specification. MSCs are known to secrete these specific growth factors.

Thus, we hypothesized that MSCs change the fiber type composition in regenerating skeletal muscle towards fast-MHC-positive fibers after local transplantation. The presence of fast, glycolytic fiber types assures an early supply of maximum forces.

Material and Methods

Animals

20 male Sprague Dawley rats (140–160 g, Charles River, Sulzbach, Germany) were included in this study. Animals were kept with free access to food and water at a constant temperature. The experiments were performed in accordance to the policies and principles established by the Animal Welfare Act, the NIH Guide for Care and Use of Laboratory Animals, and the national animal welfare guidelines.

Experimental Protocol

Bone marrow aspirations were taken from both tibiae and further cultured for autologous MSC therapy [5–7]. 14 days later, the left soleus muscle of each animal was bluntly crushed. In group 1 ($n = 10$) 2.5×10^6 MSCs were transplanted into the injured muscle 7 days after trauma. Group 2 ($n = 10$) served as control group (application of saline solution 7 days after trauma). In vivo functional muscle testing was performed 28 days after injury. Soleus muscles were explanted and processed for histological analysis.

Cell Harvest and Culture

Bone marrow biopsies were performed as described before [5–7]. In brief, following anesthetization of the animals bone marrow aspirations were taken from both tibiae and transferred into 10 ml Dulbecco's Modified Eagle Medium (DMEM; Gibco, Paisley, UK) containing 10% fetal calf serum (Biochrome, Berlin, Germany) and 1% penicillin-streptomycin (Sigma, Taufkirchen, Germany) cooled at 4 °C. Biopsies were seeded in 75 cm² cell culture flasks (Falcon, Heidelberg, Germany) and cultured at 37 °C and 5% CO₂. Culture medium was exchanged every 2 days. Adherent cells were transferred to 300 cm² cell culture flasks when reaching 60% confluence of the cellular layer using 0.25% trypsin (Sigma). Adherent cells were cultivated over three passages maximum. FACS analysis was performed before transplantation using the following antibodies: mouse α -rat CD44 (AbDSerotec, Kidlington, UK), mouse α -rat CD45 and mouse α -rat CD90 (both Acris Antibodies, Herford, Germany), mouse α -rat CD73 and rat α -mouse IgG (both BD Biosciences, Heidelberg, Germany). The differentiation potential was tested for osteogenic and adipogenic differentiation potential (osteogenic medium: DMEM supplemented with 200 μ mol/l ascorbic acid, 7 mmol/l β -glycerophosphate and 0.01 μ mol/l dexamethasone; adipogenic medium: DMEM supplemented with 1 μ mol/l dexamethasone, 2 μ mol/l insulin, 200 μ mol/l indomethacin and 500 μ mol/l isobutylmethylxanthine).

Skeletal Muscle Trauma

Upon anesthetization of the animals, the left leg was shaved, disinfected, and an open crush injury of the left soleus muscles was performed as described before [5–7]. In brief, soleus muscles were crushed using a curved artery forceps (Aesculap, Tuttlingen, Germany; closing pressure: 112 ± 5.1 N, data obtained from the material testing device Zwick 1455 (Zwick GmbH, Ulm, Deutschland)) three times distally and two times proximally to the insertion of the neurovascular bundle.

MSC Preparation and Transplantation

Adherent cells were detached using 0.25% trypsin, centrifuged, washed twice with phosphate-buffered saline (PBS), and re-suspended in 20 μ l 0.9% saline solution. MSCs were injected into the soleus muscle through a 25-gauge needle 7 days after trauma (group 1). Injections with 20 μ l of saline were performed as sham procedure (group 2).

Biomechanical Evaluation

In vivo muscle force testing was performed as previously described [5–7]. In brief, the sciatic nerve and the soleus muscle were dissected and the tendon of the soleus muscle separated from the Achilles tendon. The soleus muscle tendon was attached to a force transducer (Experimetria, Budapest, Hungary) with vicryl suture. A pre-tension of 0.15 N was applied to the muscle and then the sciatic nerve was stimulated bipolarly

Fig. 1. Longitudinal section of a traumatized soleus muscle following MSC transplantation after staining against fastMHC. The gray shaded area depicts the origin of a 10-fold magnification of the section (framed area).

Fig. 2. Longitudinal section of a healthy soleus muscle after staining against fastMHC.

(fast twitch: 5 pulses at 9 mA / 75 Hz, duration 0.01 s; tetany: 5 pulses at 9 mA / 75 Hz, duration 3 s; intervals 5 s). Upon measurements animals were sacrificed by intracardiac potassium injection.

Histological Analysis

The fixated muscles were dehydrated, embedded in paraffin, and sectioned longitudinally.

Sirius Red Staining for Connective Tissue Evaluation

Deparaffinized and rehydrated sections were incubated for 60 min in Sirius Red solution (5 g sirius red (Fluka, Direct Red 80 Number 43665, Sigma) diluted in 500 ml saturated picric acid followed by two washes in 0.5% acetic acid. Dehydration in ascending alcohol series and a final application of xylol (2 × 5 min) was performed. To analyze the total area of endo- and perimysial fibrosis, images of whole longitudinal sections of the muscle were recorded using a Leica DMRB light microscope (Leica, Wetzlar, Germany) equipped with an AxioCam MRc (Carl Zeiss, Göttingen, Germany). Pictures were analyzed using the software KS 400 Version 3.0 (Carl Zeiss). The absolute area of red connective tissue was measured.

Fast- and Slow-Myosin Staining for Fiber Type Evaluation

Deparaffinized and rehydrated sections were fixed in acetone for 10 min and washed in PBS twice for 2 min each. Next, sections were incubated in 2% horse serum (Vector, Eching, Germany) diluted in 10% PBS for 30 min at room temperature. Then the primary antibody was applied (anti-myosin fast, clone My 32 (1:4000, #M4276) or monoclonal mouse anti-myosin slow (1:10,000, #M8421, Sigma), for 1 h at 37 °C. Following washing twice with PBS for 5 min, the secondary antibody was applied (anti-mouse, rat adsorbed, biotinylated, made in horse, 2% antibody di-

luted in 2% horse serum in 10% PBS; Vector). Next, sections were washed twice with PBS for 5 min and the avidin-biotin-complex was applied for 50 min at room temperature (AP-Standard-Kit AK 5000; Vector). Finally, sections were washed twice with PBS for 5 min, and a nucleus staining (Mayers Haemalaun method) was performed. Whole longitudinal sections of the muscle were recorded and analyzed as described above (see section 'Sirius Red Staining for Connective Tissue Evaluation'). The absolute areas of fastMHC and slowMHC were measured (fig. 1, 2).

Statistical Analysis

The median and range were determined for each measure. A statistical significance analysis was performed using the non-parametric Mann-Whitney U test for independent samples for comparisons between the treatment and the control group. The level of significance was set to 0.05.

Results

Comparing traumatized left soleus muscles intra-individually against the contralateral healthy soleus muscles, the transplantation of MSCs improved muscle forces significantly after fast-twitch stimulation (group 1: 0.76 (0.51–1.15), group 2: 0.45 (0.32–0.73); $p = 0.01$). Tetanic stimulation also led to a significantly increased force development following MSC transplantation (group 1: 0.63 (0.4–1.21), group 2: 0.34 (0.16–0.48); $p = 0.04$).

Healthy muscles showed a very low amount of fibrotic tissue (1.38 (0.62–2.87 mm²). The amount of fibrosis was not influenced by the treatment with MSCs (group 1 vs. group 2 $p = 0.42$; fig. 3).

Analysis of fiber type distribution in healthy soleus muscles showed a percentage of slowMHC of 87.52% (83.05–96.35%) and of fastMHC of 12.47% (3.65–16.95%) (slowMHC vs. fastMHC; $p = 0.001$, fig. 2). Histologic analysis showed an increase of fastMHC-positive fibers in square millimeters and percent following MSC treatment (group 1 vs. group 2; mm²: $p = 0.01$, percent: $p = 0.007$, fig. 1, 4).

In MSC-treated rats, animals exhibited no significant difference for the area of slowMHC-positive muscle fibers (group 1 vs. group 2, $p = 0.66$). An overview of the histologic analysis of fastMHC and slowMHC fibers is given in table 1.

Discussion

Earlier studies have shown that MSC treatment in muscle crush trauma apparently allows enhancing muscle regeneration, but the underlying mode of action or structural change have remained unclear so far. The present study aimed at revealing the structural change due to MSC transplantation with special regard to the fiber type composition in the soleus muscle of the rat. We could demonstrate that the composition of the slowMHC and fastMHC fibers is changing following MSC transplantation. Apparently, MSC treatment leads to a structural change towards an increased ratio of fastMHC fibers

Fig. 3. Fibrotic area following open crush trauma in rat soleus muscles of animals treated with sodium chloride solution (NaCl) or MSCs (Cell). Fibrosis was assessed using Picro Sirius Red staining. p value = 0.42.

Fig. 4. Percentage of fastMHC-positive fibers in rat soleus muscles of animals treated with sodium chloride solution (NaCl) or MSCs (Cell). p value = 0.007.

over the slow ones. Moreover, the transplantation led to an improved regeneration of muscle force. Therefore, the described fiber type shift could represent a possible mode of action of MSCs to regenerate muscle substance.

Mammalian skeletal muscle contains different types of fibers that are distinguishable by their expression patterns of specific isoforms of MHC. Principally, a differentiation can be made between slow contracting fibers (type 1, slowMHC),

which are fatigue resistant and therefore ideal for low-intensity, long-lasting contractions, and fast contracting fibers (type 2, fastMHC), showing high velocity in shortening and low resistance to fatigue. Since muscle power is also dependent on the speed of shortening, the maximum power is lower in slow-MHC fibers than in fastMHC fibers [18, 19]. FastMHC fibers can further be subdivided into three subsets: type 2A, 2X and 2B [19].

Table 1. Histologic analysis of fiber type distribution

	Treated	Untreated	p value
FastMHC-positive area, mm ²	5.12 (0.73–26.47)	2.43 (0.14–9.57)	0.01*
FastMHC-positive area, %	24.52 (8.19–51.89)	17.23 (2.84–76.01)	0.007*
SlowMHC-positive area, mm ²	14.66 (1.73–32.58)	15.62 (0.48–35.49)	0.66
SlowMHC-positive area, %	75.47 (48.11–91.81)	82.76 (23.99–97.16)	0.007*
*Indicates significant difference. Values given as median (range).			

In the current investigation, the transplantation of MSCs led to a significant increase in muscle contraction force (fast-twitch $p = 0.01$; tetany $p = 0.04$). Histologic analysis of MSC-treated animals showed no reduction of the formation of fibrosis ($p = 0.42$). It is known, that MSCs secrete matrix-metalloproteinases (MMPs) that are able to digest collagen fibers, which triggered the hypothesis that a reduction in fibrosis would take place. Nguyen et al. [20] observed a reduction of fibrous tissue and apoptosis after the injection of MSC-derived growth factors following myocardial infarction. Maybe the number of 2.5×10^6 transplanted MSCs had been too low in opposition to the extensive crush trauma, and could therefore not cause a measurable amount of reduction in the histologic analysis. A possible approach might be the evaluation of digestion products on a molecular level.

The histological analysis of the fiber types was performed 4 weeks after trauma in this study in order to be able to correlate the histological results with the biomechanical evaluation of the contraction force measurements. The investigated healthy soleus muscle consisted of 87.52% (83.05–96.35%) of slowMHC fibers, which corresponded with previously published data [21]. The absolute amount (expressed in mm²) of fastMHC-positive fibers increased significantly ($p = 0.01$) in animals treated with MSCs while the amount of slowMHC fibers remained unchanged ($p = 0.66$). When calculating the percent ratio of both fiber types, the histologic analysis revealed a significant increase of fastMHC and a decrease of slowMHC fibers following MSC treatment ($p = 0.007$).

The regenerating myofibers initially express neonatal MHCs. This is followed by the development of adult fastMHCs. In a third step the conversion from fast to slowMHC fibers occurs. While the first switch (neonatal $\rightarrow$ fastMHC) is described as a ‘default’ program, which occurs without innervation, for the second switch the presence of a connection to the motoneuron is crucial [22]. Jercovic et al. [23] found gene expressions neither for slow nor for fastMHC until day 3 after a myotoxin trauma in rat soleus muscles. The first detectable MHC isoforms derived from an embryonic or neonatal stadium. FastMHC transcripts were the first adult MHC isoforms and were detected at day 4 in both innervated and denervated muscles. They remained the predominant transcripts in denervated muscles. In innervated muscle fastMHCs were down-regulated by day 5 and rested at a low level after day 7. Motoneurons are able to re-program gene expression of mus-

cle fibers. In a previous study using the same trauma model, we could show the formation of newly developed neural endplates, indicating a re-innervation of denervated and segregated regenerating myofibers [24]. Following cross-re-innervation (i.e., innervation of slowMHC fibers with ‘fast’ motor neurons and innervation of fastMHC fibers with ‘slow’ motor neurons), slowMHC fibers tend to express fastMHC and vice versa [19]. Mendler et al. [25] found a slow-to-fast fiber type transition in re-innervated soleus and could show a persistent change towards a fastMHC phenotype in regenerated muscles. In contrast, Maatsura et al. [17] observed a fastMHC to slowMHC fiber type shift 2 weeks after the trauma, which persisted thereafter. The trauma model was based on cardiotoxin. The authors believe that a chemical trauma model does not necessarily reflect a clinically relevant situation. Furthermore, it is not comparable to the crush trauma model used in the presented study.

Future investigations have to evaluate the fiber type composition at later time points to analyze if the switch towards fast fibers is re-converting to the physiological fiber type pattern of the rat soleus muscle. According to Mendler et al. [25], it could also be possible that an initial cross-innervation leads to a persistence of the observed fast fiber type shift.

Conclusion

The analysis of the fiber distribution revealed a new and so far unknown mode of action of the transplanted cells in a crush muscle trauma. Muscles that underwent MSC transplantation developed a higher amount of fastMHC fibers compared to untreated animals. In line with Maatsura et al. [17] the authors believe that such experiments can help to gain a general understanding on muscle regeneration, a field in which research has just begun to unravel the mechanisms involved in muscle fiber type composition. Further investigations on a molecular and protein level are needed to get a deeper understanding of the underlying mechanisms.

Disclosure Statement

The authors have nothing to disclose.

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