ORIGINAL ARTICLE

Functional Overloading of Dystrophic Mice Enhances Muscle-Derived Stem Cell Contribution to Muscle Contractile Capacity

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ABSTRACT. Ambrosio F, Ferrari RJ, Fitzgerald GK, Carvell G, Boninger ML, Huard J. Functional overloading of dystrophic mice enhances muscle-derived stem cell contribution to muscle contractile capacity. Arch Phys Med Rehabil Arch Phys Med Rehabil 2009;90:66-73.

Objectives: To evaluate the effect of functional overloading on the transplantation of muscle derived stem cells (MDSCs) into dystrophic muscle and the ability of transplanted cells to increase dystrophic muscle's ability to resist overloading-induced weakness.

Design: Cross-sectional.

Setting: Laboratory.

Animals: Male mice (N=10) with a dystrophin gene mutation.

Interventions: MDSCs were intramuscularly transplanted into the extensor digitorum longus muscle (EDL). Functional overloading of the EDL was performed by surgical ablation of the EDL's synergist.

Main Outcome Measures: The total number of dystrophin-positive fibers/cross-section (as a measure of stem cell engraftment), the average number of CD31+ cells (as a measure of capillarity), and in vitro EDL contractile strength. Independent *t* tests were used to investigate the effect of overloading on engraftment, capillarity, and strength. Paired *t* tests were used to investigate the effect of MDSC engraftment on strength and capillarity.

Results: MDSC transplantation protects dystrophic muscles against overloading-induced weakness (specific twitch force: control 4.5N/cm^2±2.3; MDSC treated 7.9N/cm^2±1.4) (P=.02). This improved force production following overloading is concomitant with an increased regeneration by transplanted MDSCs (MDSC: 26.6±20.2 dystrophin-positive fibers/cross-section; overloading + MDSC: 170.6±130.9 dystrophin-positive fibers/cross-section [P=.03]). Overloading-induced increases in skeletal muscle capillarity is significantly correlated with increased MDSC engraftment (R^2=.80, P=.01).

Conclusions: These findings suggest that the functional contribution of transplanted MDSCs may rely on activity-dependent mechanisms, possibly mediated by skeletal muscle vascularity. Rehabilitation modalities may play an important role in the development of stem cell transplantation strategies for the treatment of muscular dystrophy.

Key Words: Contractile function; Duchenne muscular dystrophy; Skeletal muscle; Stem cells.

UNLIKE MANY OTHER FORMS of adult tissue, healthy skeletal muscle displays a remarkable ability for regeneration after injury. This regenerative capacity relies primarily on a population of progenitor cells, muscle satellite cells, which reside between the basement membrane and the sarcolemma of muscle fibers.[1] After injury, these normally quiescent satellite cells become activated, proliferate, and fuse to form myofibers.[2] Both satellite cell activation and proliferation are necessary for skeletal muscle regeneration. Muscles that show declines in the absolute numbers of satellite cells, as seen with aging[3,4] and DMD,[5,6] are also characterized by a decreased regenerative capacity.

In healthy muscle, the protein dystrophin helps maintain membrane integrity during muscle contraction. In dystrophic muscle, an X-linked mutation in the dystrophin gene leads to a critical or complete loss of this membrane-bound protein.[7-10] Dystrophic muscle fibers have a decreased resistance to muscle injury and become inflamed, necrotic, and fibrotic, ultimately

List of Abbreviations

CSA	cross-sectional area
DS	donkey serum
DMD	Duchenne muscular dystrophy
mdx	dystrophin gene mutation
EDL	extensor digitorum longus
GFP	green fluorescent protein
½RT	half relaxation time
HS	horse serum
Lo	optimal length
MDSC	muscle-derived stem cell
OL	overload
PBS	phosphate-buffered saline
Pt	peak twitch tension
Po	peak tetanic tension
TPT	time to peak twitch tension
VEGF	vascular endothelial growth factor

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resulting in severe muscle wasting and muscle fatigue.[11,12] Therefore, dystrophic muscle becomes unable to tolerate the loading typical of everyday activities. Clinically, the decreased resistance to mechanical loading of dystrophic muscle is manifest by severe muscle weakness that renders children with DMD unable to ambulate by their early teens.

Although the past 20 years have seen significant efforts dedicated to developing biologic and cell-based therapies to ameliorate the decreased muscle function seen in DMD, there is currently no effective treatment available. Investigations performed in animal models of DMD provide important insights for a greater understanding of the pathogenesis of this disorder in humans and a vehicle for evaluating the efficacy of regenerative medicine approaches.[13]

Among the most promising methods investigated, the transplantation of healthy, activated satellite cells (myoblasts) into dystrophic mouse skeletal muscle offers the possibility of restoring the dystrophin protein into the host muscle. Transplanted myoblasts are believed to fuse together, thereby forming new, dystrophin-positive fibers, or fuse with damaged host myofibers, thereby aiding in regeneration. However, this treatment has been met with limited success, in part because of the massive cell death after transplantation of myoblasts.[14,15] MDSCs, on the other hand, appear to show an enhanced transplantation capacity, primarily because of their strong capacity for self-renewal, their ability to undergo multipotent differentiation, their immune-privileged behavior, and their ability to survive because of an increased resistance to stress.[16-18] These characteristics may favor the survival of the MDSCs after transplantation and, therefore, their use in the development of biologic therapies. Previous results performed in animal models of dystrophy show that the transplantation of MDSCs may, at least partially, restore normal histology of female dystrophic muscle for up to 3 months after transplantation.[16] In humans, the intramuscular transplantation of MDSCs has been shown to be safe and feasible.[19] Still undetermined, however, is the contribution of these engrafted stem cells to the overall physiologic functioning of the host muscle and how well transplanted stem cells respond to increased mechanical demands, such as muscle loading.

Healthy skeletal muscle is known to hypertrophy in response to overloading and stretching. It is widely accepted that environmental cues resulting from muscle injury or exercise stimulate resident satellite and stem cells to proliferate and differentiate to form multinucleated myotubes. However, how exercise and/or mechanical loading affect the proliferation and differentiation of transplanted stem cells has received much less attention. In the face of increased muscle degeneration resulting from overloading of dystrophic muscles, are transplanted MDSCs able to enhance muscle regeneration and ultimately improve muscle functioning?

The purpose of this study was to evaluate the effect of functional overloading on the efficacy of MDSCs transplantation and to investigate a possible role of skeletal muscle vascularity on stem cell engraftment. In addition, we investigated whether transplanted stem cells were able to increase dystrophic muscle's ability to withstand functional overloading.

METHODS

Animals

A total of 10 mdx mice, an animal model of DMD (C57BL/10ScSc-DMD^{mdx}), that were 6 months of age were used in this study (fig 1). Animals were obtained from Jackson Laboratories, Bar Harbor, Maine, or were bred in-house. Mdx mice lack the protein dystrophin because of a spontaneously occurring point mutation in the dystrophin gene.[20] These animals not only provide a useful model of muscular dystrophy but also provide a working model for tracking the fate of transplanted stem cells. Because engrafted donor cells differentiate into myotubes and muscle fibers expressing dystrophin, they can be easily distinguished from host cells. All animal procedures used in this study were approved by the Institutional Animal Care and Use Committee of Children's Hospital of Pittsburgh.

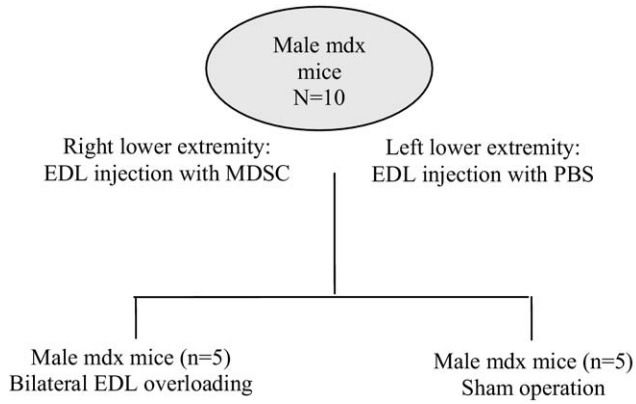

Fig 1. An animal grouping schematic.

Muscle-Derived Stem Cells Isolation

MDSCs were isolated from skeletal muscle biopsies obtained from female 3-week-old healthy mice by using a previously described modified preplate technique.[16,17] MDSCs were cultured in normal growth medium consisting of Dulbecco's Modified Eagle Medium 11995-073[a] supplemented with 10% fetal bovine serum, 10% HS, 1% penicillin/streptomycin, and 0.5% chick embryo extract. The MDSCs were grown and maintained in growth medium to approximately 30% confluence and were subsequently passaged.

On the day of injection, MDSCs were bathed in 20% trypsin and spun at 2000rpm for 5 minutes. The resulting pellet was resuspended in PBS + 0.1% microsphere beads solution at a concentration of 1.0 to 1.4 $\times$ 10^7 cells/mL.

Muscle-Derived Stem Cells Transplantation and Muscle Overloading

All animals were anesthetized with 2% isoflurane administered by inhalation. Bilateral lower extremities were shaved, and the skin was sterilized with iodine. An anterior longitudinal skin incision was performed to permit exposure of the entire anterior crural muscles. Right-side EDL muscles were injected locally with 0.7 to 1.0 $\times$ 10^5 MDSCs suspended in 7 μL PBS + 0.1% green fluorescent microsphere beads. Left-side control EDLs were injected with an equal volume of PBS + 0.1% green fluorescent microsphere beads. The track of the needle was oriented along the direction of the EDL muscle fibers for injection.

To induce muscle hypertrophy, we used tenotomy of the synergistic muscle, which increases the loads applied on the intact muscle. Bilateral EDL muscles of the animals in the OL group (n=5) were functionally overloaded by surgical ablation of the tibialis anterior muscle, as described by Tsika et al.[21] EDL functional overloading has been previously shown to result in a decreased twitch and tetanic tensions of dystrophic muscles of comparably aged animals.[22] Briefly, at the time of MDSC or PBS injection, the tibialis anterior muscle was ex-