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Mini Review

Cell therapeutic approaches using multipotent mesenchymal stromal cells for muscular dystrophy

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Multipotent mesenchymal stromal cells (MSCs) have potential therapeutic uses owing to their ability to differentiate *in situ* into various cell types with immunosuppressive properties. Clinically, MSCs have been used to treat inflammatory diseases, such as steroid-resistant graft-versus-host disease. We previously reported a strategy to expand MSC cultures and to induce these cells to undergo myogenic differentiation, which is promising for the treatment of muscular diseases. Muscular dystrophy is an incurable genetic disease with early mortality and causes skeletal muscle weakness with chronic inflammation. Here, we focused on the beneficial properties of MSCs, namely, they can undergo mesoderm differentiation, have the ability to fuse with dystrophic muscles, and have anti-inflammatory activities. In this review, we highlight and discuss MSC-based therapeutic approaches for muscular dystrophy.

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Introduction

Multipotent mesenchymal stromal cells (MSCs) from bone-marrow are conventionally defined as adherent non-hematopoietic cells that express several cell surface antigenic markers, such as CD44, CD73, CD90, and CD105, but not the hematopoietic markers CD34 or CD45[1)]. Although they were originally identified in bone-marrow[1)], MSCs can be extracted from numerous tissues including

adipose tissue[2], heart tissue[3], Wharton's jelly[4], dental pulp[5], peripheral blood[6], cord blood[7], menstrual blood[8-10], and fallopian tube tissue[11]. MSCs have been extensively studied owing to their ability to self-renew and differentiate into many different cell types in culture, particularly cells of mesodermal origin such as osteoblasts, chondrocytes, adipocytes, and myocytes[12, 13]. Furthermore, MSCs can also differentiate into cells of non-mesodermal origin, such as hepatocytes[14, 15], neural cells[16], and epithelial cells[17]. MSCs can influence immune effector cell development, maturation, and function, as well as alloreactive T-cell responses, through the production of bioactive cytokines and proteins[18]. The multilineage potential of MSCs has been further exploited in their potential use as therapies for various diseases, which is feasible because these cells can be readily obtained from patients, are easily expanded in culture, and are not tumorigenic. Furthermore, the use of third-party MSCs in cell therapies is facilitated by the fact that these cells are immunoprivileged because they do not express human leukocyte antigen (HLA) class II proteins, CD40, CD80, or CD86[19], and express only low levels of HLA class I proteins. Consequently, MSCs are not lysed by natural killer (NK) cells or cytotoxic T lymphocytes[20].

Recently, MSCs have obtained market authorization as a product for the treatment of acute graft-versus-host disease (GVHD) in Canada and New Zealand. MSCs are being evaluated in Phase 3 clinical trials to treat Crohn's disease and acute radiation syndrome, and in Phase 2 trials to treat several ailments such as type I diabetes, acute myocardial infraction, and pulmonary disease (Mesoblast Inc., http://www.mesoblast.com/products/overview). Furthermore, MSCs are extremely attractive candidates for cell-based strategies that target other diseases such as muscular disease[21]. In this review, we discuss how MSC therapy might have beneficial effects on the progression of muscular dystrophy via eliciting anti-inflammatory effects and/or promoting the regeneration of myofibers.

Myogenic differentiation of MSCs

MSCs themselves can be induced to differentiate along the myogenic pathway, thereby fusing with myotubes and promoting the formation of new muscle fibers after being transplanted into muscle tissue[22].

MSCs can form muscle cells after treatment with one or a combination of 5'-azacytidine (a demethylating agent), hydrocortisone[23], dexamethasone, ascorbic acid, and growth factors, when co-cultured with immortalized myoblast cells (C2C12)[24, 25], or when exposed to the conditioned media of these cells[26]. A method has been reported to induce skeletal muscle lineage cells from human and rat adherent MSCs via transduction with the Notch1 intracellular domain and administration of certain trophic factors and cytokines[22]. Upon genetic modification with a lentiviral vector encoding Pax3, which is the master regulator of the embryonic myogenic program, expression of myogenic regulatory factors is activated in human MSCs after 4 weeks of culture, suggesting that Pax3 enables MSCs to differentiate into myogenic progenitors *in vitro*[27].

We have also reported efficient methods to expand MSC cultures obtained from dog bone-marrow and to induce the myogenic differentiation of these cells[21]. CD271 is a marker of progenitor cells and bone-marrow-derived MSCs[28]. MSC cultures enriched in $CD271^+$ cells grow better than CD271-depleted cultures. Transduction of $CD271^+$ MSCs with MyoD-expressing adenovirus vector, as an inducer of myogenic differentiation, causes the formation of myotubes that express late myogenic markers. These methods may be useful to efficiently transplant cells for the treatment of muscle disease.

Paracrine effects of MSCs

MSCs secrete distinctively different cytokines and chemokines, such as greater amounts of VEGF-α, IGF-1, EGF, keratinocyte growth factor, angiopoietin-1, stromal derived factor-1, macrophage inflammatory protein-1α and β and erythropoietin[29]. After transplantation, MSCs home to interstitial muscle tissue and localize close to satellite cells. MSCs induce the myogenic differentiation of neighboring satellite cells, as evidenced by the finding that isolated cells from muscle in which MSCs have engrafted show high myogenic activity and displayed $CD45^-$, $sca\text{-}1^-$, $Mac\text{-}1^-$, $CD34^+$, $CXCR4^+$, $\beta1\text{-integrin}^+$ characteristics[30]. Clinical interest in the application of MSCs in cell therapies is not only owing to their ability to differentiate, but also to their release of cytokines into the surrounding environment, which modifies the developmental fate of neighboring cells in a paracrine manner.

Cell therapeutic approach for muscular dystrophy

In this section, we discuss whether the myogenic differentiation of MSCs beneficially affects the progression of muscular dystrophy. Muscular dystrophy patients exhibit skeletal muscle damage that is associated with chronic

inflammation, numerous centrally nucleated fibers, and continuous cycles of myofiber degeneration/necrosis and regeneration. In particular, Duchenne muscular dystrophy (DMD) is a severe X-linked muscle disease in which mutations in the gene encoding the cytoskeletal protein dystrophin result in destruction of the dystrophin-glycoprotein complexes of the sarcolemma[31, 32]. The resulting alterations in mechanical and signaling functions contribute to membrane fragility, necrosis, and immune cell infiltration, and cause progressive degeneration of striated muscle. The pathology of DMD muscles leads to chronic inflammation, fibrosis, fat infiltration, and impaired vasoregulation, manifesting as muscle weakness and eventually skeletal muscle atrophy[33, 34]. As the disease progresses, wheelchairs and ventilatory assistance are required, and patients often succumb to cardiac dysfunction and respiratory failure[35].

Cell-based therapies for DMD have the potential to restore dystrophin expression and, therefore, also the parenchyma of muscle. The following section introduces the general concepts behind gene- and cell-based strategies to treat DMD. Transplantation of mesoangioblasts, hematopoietic stem cells, myoblasts, and muscle-derived stem cells has been examined as a possible strategy to treat DMD and as a system to deliver therapeutic recombinant proteins to target muscle tissues[36-38].

In dystrophin-deficient *mdx* mice, transplanted human MSCs were incorporated into myofibers and dystrophin expression was subsequently restored[22, 39, 40]. MSCs transduced with Notch1 and treated with trophic factors and cytokines can differentiate when transplanted into the degenerated muscles of rats and *mdx*-nude mice. The induced population contains Pax7$^+$ cells that contribute to the subsequent regeneration of muscles[22]. Transplantation of human adipose-derived MSCs transduced with a MyoD-coding lentiviral vector into the injured muscles of immunodepressed Rag2$^{-/-}\gamma C^{-/-}$ mice resulted in a substantial increase in the number of myofibers and restoration of dystrophic expression[41]. Although the engraftment of human MSCs from bone-marrow is improved in the presence of Pax3, supported by an approximately 1.3-fold increase in the level of myofibers in immunodepressed mdx mice, this engraftment is not accompanied by functional recovery[27].

In therapeutic approaches using medium-sized animal models of DMD, such as dogs, transplantation of heterologous mesoangioblasts in golden retriever muscular dystrophy (GRMD) ameliorated and preserved active motility[42]. However, the development of an analogous approach for clinical use in humans has been hindered by the inability to overcome several obstacles, including poor cell survival rates, limited dissemination of injected cells, immune responses to allogeneic cells, the presence of the neotransgene product in dystrophic muscles, and the inability to specifically target the cells to particular regions, such as cardiac tissue[43].

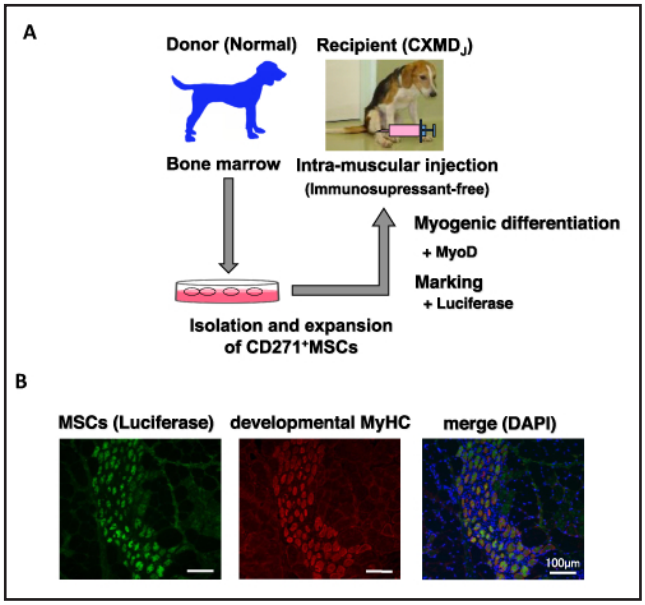

Fig.1 Successful long-term engraftment and myogenic differentiation of CD271$^+$ MSCs

(A) Transplantation procedure. CD271$^+$ mesenchymal stem cells (MSCs) were enriched from the bone-marrow of healthy dogs and expanded. These cells were transduced with a luciferase-expressing lentivirus vector as a marker and a MyoD-expressing adenoviral vector, and injected into Duchenne muscular dystrophy dogs without immunosuppressants. (B) Engraftment and differentiation. At 12 weeks after CD271$^+$ MSCs injection, cryosections from the muscle of recipients were stained with antibodies specific for luciferase expressed in MSCs (green) and the myogenic marker developmental myosin heavy chain (dMyHC) (red). The merged image includes staining of nuclei with 4', 6'-diamidino-2-phenylindole (DAPI) (blue). CD271$^+$ MSCs formed muscle-like tissue that expressed dMyHC at 12 weeks after transplantation (Quote from ref. 21) with minor revision).

In our previous study, we found that wild-type CD271$^+$ MSCs in a myogenic cell lineage transplanted into dogs with X-linked muscular dystrophy in Japan (CXMDJ) formed clusters of muscle-like tissues within 8-12 weeks in the absence of immunosuppression[21] (Fig.1). In the newly formed tissues, expression of developmental myosin heavy chain, which is a marker of myogenesis, and dystrophin

was up-regulated. In CXMD$_J$, transplanted MSCs that are delivered systemically must specifically engraft into muscle tissue. MSCs normally mobilize in the blood in response to skeletal muscle injury[44], and several homing/migration/engraftment studies have suggested that MSCs delivered systemically can "home" to the site of injury[45-49]. Intra-arterial injection of CD271$^+$ MSCs results in engraftment at the site of acutely injured muscle and the formation of muscle fibers[21]. These findings suggest that a cell transplantation strategy using CD271$^+$ MSCs is a promising treatment approach for DMD.

Human immature dental pulp stem cells (hIDPSCs), which are obtained from decidual tooth tissue and comprise a homogeneous population positive for MSC markers, are a more convenient cell source than bone-marrow. These cells are similar to populations of dental pulp MSCs with immunosuppressive activity[50]. The proliferation and neurogenicity of hIDPSCs in dental pulp are more potent than those of bone-marrow MSCs, probably because the former cells are of neural crest origin. hIDPSCs inhibit the proliferation of phytohemagglutinin-stimulated T-cells, and therefore would elicit stronger effects than bone-marrow MSCs[50, 51]. After transplantation of hIDPSCs into young GRMD dogs without immunosuppression, a limited number of muscle fibers express dystrophin[52].

These approaches using MSCs need to be further developed to obtain fully differentiated muscle fibers and to stimulate functional recovery of skeletal muscles in DMD patients.

Therapeutic approach using the immunomodulatory properties of MSCs

MSCs regulate inflammation through mechanisms thought to involve the inhibition of monocyte differentiation into immature dendritic cells (DCs)[53]. This results in the skewing of DCs toward macrophages[54, 55], suppression of DC maturation[54, 56], inhibition of T-cell and B-cell proliferation, suppression of NK and cytotoxic T cell function[57], and inhibition of neutrophil apoptosis, inducing the generation of regulatory T-cells[58] (Fig.2). Therefore, the effects of MSCs on immune diseases have been investigated[59, 60]. Furthermore, interleukin (IL)-10-transfected MSCs can reduce the severity of acute GVHD and aid the recovery of cardiac function due to high levels of immunosuppression[61, 62]. Another study reported that human amniotic membrane-derived mesenchymal cells (hAMCs) markedly increase HLA-G expression *in vitro* following administration of IL-10

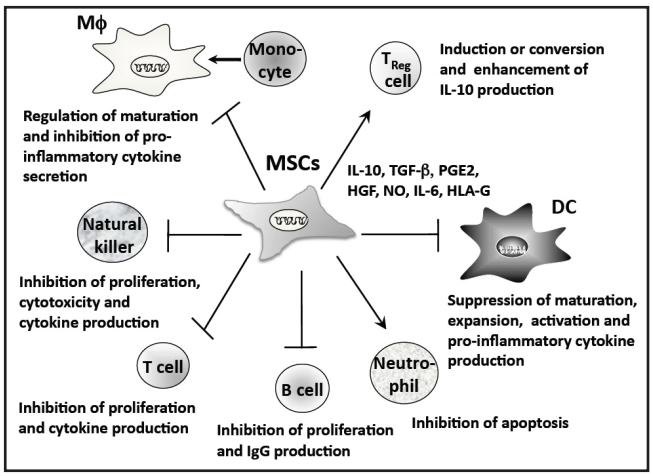

Fig.2 Immunoregulation by mesenchymal stems cells (MSCs)
The immunoregulation of various cell types by MSCs through IL-10, TGF-β, PGE2, HGF, NO, and HLA-G is shown.

or progesterone, which plays an important role in feto-maternal tolerance during pregnancy, and, surprisingly, also increases the efficiency of cardiomyogenic trans-differentiation *in vitro* and *in vivo*[63]. hAMCs have a great ability to transdifferentiate into cardiomyocytes and to acquire immunologic tolerance *in vivo*, and are, therefore, a promising source of allograftable stem cells for cardiac regenerative medicine[63].

In dystrophic muscles, activated immune cell infiltrates (e.g., T lymphocytes and macrophages) are observed during the early stages of disease and play a critical role in muscle wasting[55-60]. Depletion or inhibition of these cells significantly improves dystrophic muscle pathology[64-66]. The findings of these studies suggest that much of the muscle damage that occurs when dystrophin is deficient is caused by inflammatory cells, as well as by direct mechanical damage.

Inflammatory cytokines, serum markers, and intramuscular nuclear factor-κB are not upregulated in a δ-sarcoglycan-deficient dystrophic hamster model following intramuscular injection of human- and pig-derived MSCs. Additionally, transplantation of these MSCs is associated with the formation of new muscle fibers and reduced muscular oxidative stress[67]. However, the majority of studies using MSCs in animal models do not report a significant, if any, increase in muscle contractile force[27]. The therapeutic effects of MSCs are believed to not only be owing to their differentiation in injured tissue, but also to their production of paracrine factors that inhibit apoptosis of injected cells, induce anti-inflammatory effects, and stimulate the

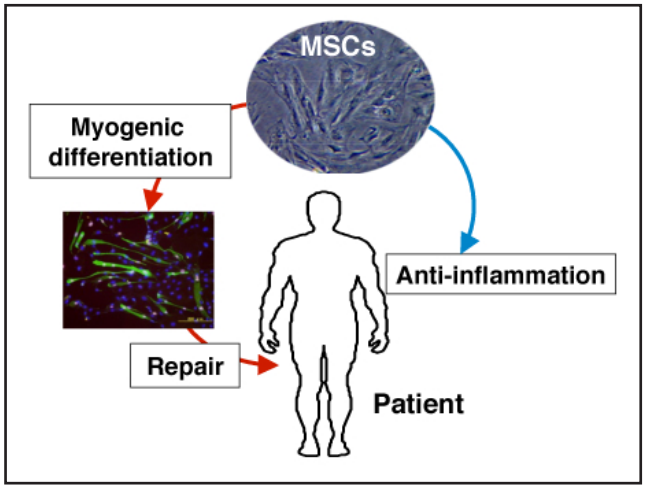

Fig.3 Cell-based therapeutic approach for muscular dystrophy
An approach to treat DMD using MSCs is shown. MSCs undergo myogenic differentiation and thereby contribute to muscle repair, and also elicit anti-inflammatory effects.

proliferation of endogenous stem cells at the site of injury.

Conclusion and future directions

Clinical interest in MSCs for cell therapeutic applications is based on their anti-inflammatory properties and their ability to release cytokines into the surrounding environment, thereby modifying the developmental fate of neighboring cells. In this review, we introduced various strategies for the engraftment of transplanted cells as a therapeutic approach for muscular dystrophies. MSCs are a promising therapy for muscle disease because they elicit immunosuppressive and/or anti-inflammatory effects and can undergo myogenic differentiation contributing to muscle repair (Fig.3).

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Disclosure of potential conflicts of interest

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