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Biological Approaches to Improve Skeletal Muscle Healing after Injury and Disease

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Abstract

Skeletal muscle injury and repair are complex processes, including well-coordinated steps of degeneration, inflammation, regeneration, and fibrosis. We have reviewed the recent literature including studies by our group that describe how to modulate the processes of skeletal muscle repair and regeneration. Antiinflammatory drugs that target cyclooxy-genase-2 were found to hamper the skeletal muscle repair process. Muscle regeneration phase can be aided by growth factors, including insulin-like growth factor-1 and nerve growth factor, but these factors are typically short-lived, and thus more effective methods of delivery are needed. Skeletal muscle damage caused by traumatic injury or genetic diseases can benefit from cell therapy; however, the majority of transplanted muscle cells (myoblasts) are unable to survive the immune response and hypoxic conditions. Our group has isolated neonatal skeletal muscle derived stem cells (MDSCs)

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that appear to repair muscle tissue in a more effective manner than myoblasts, most likely due to their better resistance to oxidative stress. Enhancing antioxidant levels of MDSCs led to improved regenerative potential. It is becoming increasingly clear that stem cells tissue repair by direct differentiation and paracrine effects leading to neovascularization of injured site and chemoattraction of host cells. The factors invoked in paracrine action are still under investigation. Our group has found that angiotensin II receptor blocker (losartan) significantly reduces fibrotic tissue formation and improves repair of murine injured muscle. Based on these data, we have conducted a case study on two hamstring injury patients and found that losartan treatment was well tolerated and possibly improved recovery time. We believe this medication holds great promise to optimize muscle repair in humans.

Keywords

muscle injury; muscle derived stem cells; fibrosis; losartan; angiotensin receptor blocker; paracrine effect

INTRODUCTION

Vertebrate skeletal muscle comprises bundles of contractile muscle fibers that are multinucleated cells formed by the fusion of muscle cells (myocytes) into syncytia. The myofibers are surrounded by a membrane (sarcolemma), and each group forms a fascicle. Fascicles are in turn surrounded by connective tissue sheath called the endomysium. Groups of fascicles are enveloped by a perimysium and make up whole muscle. The skeletal muscle also contains connective, vascular, adipose, nerve, and other cell populations.

The myogenic cells in the skeletal muscle are found at a variety of different stages of maturity. The fully differentiated myofibers are surrounded by satellite cells that are within the basal lamina but outside the sarcolemma (Mauro, 1961). These cells express paired box protein 7 (Pax7) and myogenic differentiation (MyoD) surface markers, which indicate partial differentiation down the muscle lineage (Seale et al., 2000). Satellite cells remain quiescent in the muscle until an external stimulus such as an injury occurs, at which point they re-enter the cell cycle and proliferate (Hill et al., 2003). Proliferating satellite cells differentiate into myoblasts, which can then fuse to form new myofibers (Collins and Partridge, 2005). The myoblast population, which is more differentiated than satellite cells, fuse to become mature myofibers, as previously mentioned. These cells have also been used extensively for transplantation.

Distinct from both the satellite cell and the myoblast populations are the muscle derived stem cells (MDSCs). Although MDSCs are similar to satellite cells in playing a role in skeletal muscle regeneration, they are a separate population of cells that express distinct markers and phenotypes (Deasy et al., 2001; Huard et al., 2003). MDSCs are believed to be an earlier progenitor than satellite cells, expressing stem cell markers such as cluster of differentiation 34 (CD34) and stem cell antigen 1 (Sca-1), and they have the ability to differentiate down nonmuscle lineages to contribute to repair (Qu-Petersen et al., 2002). Pax-7 and Sca-1 positive cells have not been colocalized in skeletal muscle, providing

further evidence that satellite cells and MDSCs are likely distinct populations (Zammit and Beauchamp, 2001; Fig. 1).

In the incidence of a traumatic injury or muscle damage due to a genetic disease (such as Duchenne muscular dystrophy [DMD]), the muscle goes through a complex and dynamic series of events resulting in an inflammatory phase, the activation of progenitor cells, regeneration of muscle tissue, formation of fibrosis, and varying degrees of restoration of function. These phases are detailed below.

Phases of Skeletal Muscle Injury and Repair

Acute skeletal muscle injuries are common injuries, which account for a large segment of the patients presenting to orthopaedic practitioners (Crisco et al., 1994; Woolf and Pfleger, 2003; Physicians, 2005; Carling et al., 2011). Research has shown that the natural progression of muscle injury proceeds through a highly interdependent sequence of steps, leading to the restoration of tissue architecture and function (Moyer and Wagner, 2011). However, the regenerative capacity of injured skeletal muscle is limited and very often, fibrotic tissue forms, delaying the muscle's full functional recovery and predisposing it to recurrent injury (Li and Huard, 2002). Clinical findings reveal a high recurrence rate of skeletal muscle strain injuries, approaching 30% among some professional-level athletes (Woods et al., 2004). Numerous investigations have led to the identification of the molecular events and cellular transformations following muscle injury; however, the clinical treatment of this common condition still relies on conventional therapies of rest, ice, and antiinflammatory medications, which have a limited efficacy in preventing or treating the formation of posttraumatic muscle fibrosis (Almekinders, 1993; Jarvinen and Lehto, 1993; Worrell, 1994). Research conducted by our group and others showed that injured muscle undergoes a sequential process of healing phases, including muscle degeneration/inflammation, regeneration, and fibrosis (Huard et al., 2002; Li and Huard, 2002; Li et al., 2004). These phases of muscle healing can be modulated by different biological approaches that will be detailed in the sections later (Fig. 2).

MUSCLE INFLAMMATION

Muscle degeneration and concomitant inflammation begins in the first few days postinjury. Resident macrophages are activated, releasing chemoattractants, leading to the recruitment of neutrophils and monocytes. Subsequently, inflammatory mediators such as tumor necrosis factor alpha (TNFα) are released, and immune, myogenic, and fibroblastic cell interactions are coordinated. This reaction can persist for several days depending on the severity of the injury (Moyer and Wagner, 2011).

Antiinflammatory drugs are often prescribed to relieve pain after muscle injury. However, the effect of these drugs, especially nonsteroidal antiinflammatory drugs (NSAIDs) on the skeletal muscle healing remains controversial. To examine the role NSAIDs play in the process of muscle healing, our group had performed two studies to determine the effect that cyclo-oxygenase-2 (Cox-2) has in modulating muscle recovery (Shen et al., 2005, 2006). In vitro experiments showed that a Cox-2-specific inhibitor (NS-398) slows the proliferation and maturation of differentiated myogenic precursor cells and thus delays the regenerative

myogenesis process. Other investigators have found similar results using the Cox-2 selective inhibitor SC-236 (Bondesen et al., 2004, 2006). Our results thus indicate that NS-398 may hamper skeletal muscle healing. We examined the in vivo effect of NS-398 on skeletal muscle healing in a muscle laceration mouse model at different time points up to 4 weeks postinjury. The in vivo data were in agreement with in vitro results and showed delayed muscle regeneration at early time points after injury in mice treated with NS-398. Treating lacerated muscles with NS-398 led to the expression of higher levels of transforming growth factor-β1 (TGF-β1) than the untreated control muscles, and the lacerated muscles treated with NS-398 showed higher fibrosis deposition than the controls. As expected, we found fewer neutrophils and less macrophage infiltration in the muscles treated with NS-398. These results indicate that the inhibitory effect of NS-398 on the inflammatory responses delays skeletal muscle healing after laceration. Furthermore, we analyzed muscle healing following laceration injury on the tibialis anterior (TA) muscles of COX-2−/− mice and control wild type (Shen et al., 2005) by examining the histology and function of TA muscles at 5 and 14 days after injury. COX-2−/− mice TA muscles showed decreased regeneration relative to that observed in wild-type mice. These results demonstrate that the COX-2 pathway plays an important role in muscle healing, and consequently, the decision to use NSAIDs to treat muscle injuries warrants critical examination of evidence available. NSAIDs seem to impair the healing even in the heavily vascularized skeletal muscle tissue and probably affect the recovery of other soft tissues.

MUSCLE REGENERATION

In the first week postinjury, skeletal muscle promptly begins the regeneration process that peaks at 2 weeks and then decreases gradually at 3 to 4 weeks postinjury. Several studies have shown that growth factors play a variety of roles during muscle regeneration (Gospodarowicz et al., 1976; Inselburg and Applebaum, 1978; Linkhart et al., 1981; Florini et al., 1986; Olson et al., 1986; Allen and Boxhorn, 1989; Jennische, 1989; Jin et al., 1990; Yablonka-Reuveni et al., 1990; Anderson et al., 1991; Grounds, 1991; Harrington et al., 1992; Doumit et al., 1993; McFarland et al., 1993; Barnard et al., 1994; Coleman et al., 1995; Johnson and Allen, 1995; Jones and Clemmons, 1995; Lefaucheur and Sebille, 1995; Zdanowicz et al., 1995; Chambers and McDermott, 1996; Engert et al., 1996; Florini et al., 1996; Papadakis et al., 1996; Quinn and Haugk, 1996; Floss et al., 1997; Kurek et al., 1997; Lamberts et al., 1997; Barton-Davis et al., 1998; Damon et al., 1998; Springer et al., 1998; Tatsumi et al., 1998; Keller et al., 1999; Gowdak et al., 2000; Sheehan et al., 2000; De Deyne et al., 2002; Musaro et al., 2004; Wieteska-Skrzeczynska et al., 2011a,b). Using a mouse model, Menetrey et al. (2000) found that direct injections of insulin-like growth factor-1 (IGF-1), basic fibroblastic growth factor (bFGF), and, to a lesser extent, nerve growth factor (NGF), led to enhanced muscle healing in lacerated, contused, and strain-injured muscle at 2, 5, and 7 days after injury. Using human recombinant growth factors to treat muscle injuries has the advantage of feasibility and safety of the injection; however, the efficacy of direct injection of growth factors, in the form of recombinant proteins, is limited by the high concentrations typically required to elicit a measurable effect. Growth factors clearly have a dose-dependent effect on myoblast proliferation and differentiation in vitro. However, a series of three consecutive injections of a relatively high concentration (100 ng

for each growth factor) of NGF, IGF-1, or bFGF is usually required to achieve significant enhancement of skeletal muscle healing in mice (Barton-Davis et al., 1998; Menetrey et al., 2000; Fukushima et al., 2001; Li and Huard, 2002; Chan et al., 2003; Foster et al., 2003; Sato et al., 2003; Li et al., 2004; Negishi et al., 2005). Rapid clearance of these molecules in the blood circulation and their relatively short biological half-lives necessitate large amounts to be administered. Further studies will be needed to investigate the potential synergetic effects of the association of two or more growth factors and the potential use of controlled release particles containing the factors.

Another method that can be effective in delivering high, maintainable concentrations of growth factors to injured muscle is gene therapy. Data obtained from previous studies had demonstrated that IGF-1 is a potent growth factor for stimulating muscle regeneration and improving muscle healing in vivo after injury (Barton-Davis et al., 1998; Menetrey et al., 2000; Fukushima et al., 2001; Li and Huard, 2002; Chan et al., 2003; Foster et al., 2003; Sato et al., 2003; Li et al., 2004; Negishi et al., 2005). Based on this information, we genetically engineered an adenoviral vector to encode the gene for IGF-1 and evaluated its ability to improve muscle healing after injury (Lee et al., 2000). Myoblasts ex vivo transduced by IGF-1 adenovirus and then injected into lacerated muscles of immunocompetent mice led to improved muscle repair (Lee et al., 2000). Although we have seen improvement in muscle strength in skeletal muscle treated with myoblasts adenovirally treated to express IGF-1, fibrosis or scar tissue was detected by histology. This suggested that the enhancement of muscle regeneration by either cell or gene therapy improves muscle regeneration but may not completely eliminate fibrosis (Lee et al., 2000). These results suggest that high levels of IGF-1 secretion can be achieved, and delivery is feasible, but the functional recovery of the injured muscle remained incomplete. It is likely that IGF-1 modulates several actions including a stimulatory effect on myofibroblast proliferation, deposition of extracellular matrix (ECM), which may interfere with the ability of this growth factor, even at high concentrations, to improve muscle healing after injury (Jones and Clemmons, 1995; De Deyne et al., 2002). Overall, these results indicate that reducing muscle fibrosis is a complicated process that likely involves several cell populations and growth factors.

Muscle Regeneration after Stem Cell Transplantation

A variety of muscle cell populations have been used for cell transplantation in studies treating patients who are afflicted with DMD, a muscle disease characterized by the lack of dystrophin expression at the sarcolemma of muscle fibers (Hoffman et al., 1987). Transplantation of committed myoblasts into dystrophin-deficient muscle delivers normal myoblasts that fuse with host muscle fibers and/or among themselves and consequently restores dystrophin expression; however, this approach is hindered by numerous limitations, including limited cell spreading, immune responses, and poor survival of the transplanted cells (Karpati et al., 1989; Morgan et al., 1990; Huard et al., 1991, 1994; Mendell et al., 1995; Fan et al., 1996; Gussoni et al., 1997). Although the immune response and the low spreading capacity of the cells have been overcome, at least in part (Kinoshita et al., 1994; Vilquin et al., 1995), the low survival of the transplanted cells is still a major limitation. Indeed, numerous studies report that only a small percentage of the transplanted cells (less

than 1–5%) survive, therefore, approaches to increase cell survival postimplantation need to be optimized for myoblast transplantation therapies for DMD (Fan et al., 1996; Beauchamp et al., 1999; Hodgetts et al., 2000).

Efforts to promote donor myoblast survival initially focused on overcoming the inflammatory response (Guerette et al., 1997; Qu et al., 1998; Hodgetts et al., 2000). Myoblasts that are genetically engineered to express an inhibitor of the inflammatory cytokine, IL-1, showed an improved survival rate compared to nonengineered cells (Qu et al., 1998). By treating the host with CD4+/CD8+ to deplete antibodies, donor myoblast survival was enhanced in dystrophic animals (Hodgetts et al., 2000), and by treating the host animals with antibodies against Leukocyte function-associated molecule 1, death of the transplanted myoblasts was reduced (Guerette et al., 1997). This has led some investigators to focus their efforts on the isolation, identification, and characterization of the small subset of donor cells capable of surviving after transplantation (Baroffio et al., 1996; Beauchamp et al., 1999; Collins et al., 2007). Our efforts to isolate such a population are detailed in the following section.

MDSCs

Our group and others have isolated from mouse skeletal muscle a group of cells based on their adhesion characteristics to collagen-coated flasks. We used a modification of a method called the pre-plate technique (Qu-Petersen et al., 2002; Gharaibeh et al., 2008) to purify these cells from postnatal mouse skeletal muscles. In the preplating process, a skeletal muscle biopsy is mechanically broken and then enzymatically digested by a series of enzymes including dispase, collagenase, and trypsin. A single cell suspension is filtered and plated onto a series of collagen-coated flasks. The cells that are slowest to adhere (slowly adhering cells [SACs]) seem to proliferate very slowly at first and have a different morphology from the rapidly adhering cell fraction. After further passaging to remove other cells, including fibroblasts and myoblasts, SAC becomes highly enriched with MDSCs. Technical details of the protocol followed to isolate MDSCs are beyond the scope of this review (Gharaibeh et al., 2008). MDSCs have the potential for long-term proliferation without any significant changes to their cell characteristics, and they maintain a stable karyotype that has no significant numerical or structural abnormalities (Deasy et al., 2005). The end result of the preplate technique results is an enriched population of MDSCs, even though, it is a heterogeneous population. A marker profile for MDSCs is not very high for a single marker as one would obtain from isolation done by flow activated cell sorting (FACS), but typically MDSCs express high levels of Sca-1, as previously mentioned, very low levels of vimentin (a fibroblastic marker), low expression of desmin, and other differentiated muscle markers (Qu-Petersen et al., 2002).

We have shown that SAC repairs skeletal muscle in a more effective manner than myoblasts that tend to more rapidly adhere to collagen-coated flasks. Other investigators have shown that a subpopulation of muscle cells are slowly dividing (as determined by thymidine uptake), but when injected into dystrophic mice muscles, they undergo rapid proliferation and become major contributors to muscle repair (Beauchamp et al., 1999). Similarly, Collins et al. (2007) showed that among aged muscle satellite cells, there is also a subset of cells that

survive the effects of aging, and this minority population is responsible for muscle regeneration while the majority of cells progress to apoptosis. Recent studies have used FACS to separate cells based on normally expressed cell-surface markers and showed that skeletal muscle precursor cells (SMPs) show heterogeneity (Cerletti et al., 2008; Biressi and Rando, 2010). The subfraction characterized by markers $CD45^{-}$ $Sca\text{-}1^{-}$ $Mac\text{-}1^{-}$ $CXCR4^{+}$ $\beta1\text{-integrin}^{+}$ showed a high level of muscle cell repair, while non-SMPs ($CXCR4^{-}/\beta1\text{-integrin}^{-}$) were rarely identified in the muscles after transplantation (Cerletti et al., 2008). One can speculate that these subpopulations secrete different factors and have autocrine and paracrine effects on the host skeletal muscle and other tissues. These signals probably support the survival of these progenitor cells and enhance their participation in skeletal muscle repair. Indeed, we have found in previous studies that the transplantation of MDSCs that were transduced with a retroviral vector to express NGF or directly stimulated with NGF protein, into the skeletal muscle of dystrophic mdx mice, resulted in a significantly larger engraftment with a higher number of dystrophin-positive myofibers than the transplantation of nontransduced MDSCs (Lavasani et al., 2006). Our observations of newly regenerated myofibers by the transplanted MDSCs, particularly the genetically engineered MDSCs, suggest that NGF that was released had an autocrine as well as paracrine effect on neighboring cells.

One important factor that may be involved in cell survival and tissue regeneration is the expression of antioxidants by the cells. A reduction of antioxidant levels negatively affects the regeneration index of myoblasts and satellite cells (Fulle et al., 2005; Lee et al., 2006; Urish et al., 2009), and hematopoietic stem cells (Ito et al., 2006), likely through the increased ability of the cells to survive after implantation. Our group has recently shown that the MDSCs express high levels of the antioxidants glutathione (GSH) and superoxide dismutase. These molecules likely play a critical role in the cells' ability to survive the harsh transplantation microenvironment better than myoblasts and hence increase their ability to more efficiently regenerate the tissue (Urish et al., 2009; Drowley et al., 2010). Our group has recently shown that reduction of the antioxidant level of MDSCs by diethyl maleate decreased their regeneration potential, whereas an improvement in their regenerative potential was observed after enhancing their antioxidant levels with *N*-acetylcysteine. These results show a therapeutic potential for boosting antioxidant levels of stem cells before transplantation in skeletal and heart muscles (Urish et al., 2009; Drowley et al., 2010).

Donor Cell-Mediated Skeletal Tissue Repair Paracrine Action

It is clear from numerous reports using cell therapy in animal models that cell survival, differentiation, and engraftment in host tissue are important factors in assessing the efficacy of cell therapy. At the same time, it has been demonstrated that the number of cells directly involved in tissue repair are not necessarily correlated with the improvement in function exhibited by the host tissue. This is especially true in the cardiac muscle (Payne et al., 2005). Recently, our group has found that intramuscular injection of MDSCs at 4 days postinjury greatly enhanced injured skeletal muscle healing by increasing angiogenesis and reducing scar tissue formation (Ota et al., 2011). High levels of expression of vascular endothelial growth factor (VEGF) 1 week after cell injection was correlated with increased vascularity, improved muscle regeneration, and strength at week 4 (Ota et al., 2011). Thus, it is believed

that the abilities of MDSCs to secrete different molecules and engage in autocrine and paracrine activities are determining factors of the success of these stem cells in the repair process (Gharaibeh et al., 2011). Exciting results now show that stem cell-mediated repair may occur with little, if not in the absence of, donor cell differentiation. Results indicate that repair may be due to a largely overlooked characteristic of stem cells, paracrine signaling by surviving stem cells (Gnecchi et al., 2005; Murry et al., 2006; Gharaibeh et al., 2011; Fig. 3).

Trophic signaling or release of cytokines or other signaling molecules may be a key for recruiting host cells to participate in the repair, perhaps by having an effect in the local microenvironment, and/or by inducing a systemic effect, or by mediating an inflammatory response. Several reports have shown that transplanted muscle cells may induce angiogenesis in the host tissue by expressing VEGF, but it is not fully understood whether other bioactive factors may be secreted by the cells or whether they have any immunomodulatory activity (Springer et al., 1998, 2003; Deasy et al., 2009). On a similar note, evidence indicating that antiinflammatory drugs delay muscle repair implicates inflammatory cells in the repair process as mentioned before, but the role that particular host cells play in the repair process is still unclear (Almekinders and Gilbert, 1986; Obremsky et al., 1994; Mishra et al., 1995; Shen et al., 2005).

Based on these findings, further analysis of the identity of specific molecules expressed in this paracrine activity will likely affect therapeutic strategies and indeed the need for stem cell transplantation.

FIBROSIS

Excessive formation of connective tissue between skeletal muscle fibers forms muscle scar. This usually begins between the second and third week after muscle injury and continues to increase in size over time (Li et al., 2001; Huard et al., 2002). Our findings strongly indicate that scar tissue formation leads to incomplete regeneration of injured muscle tissue. Various reports have implicated TGF-β1 in the onset of fibrosis in various tissues (Czaja et al., 1989; Okuda et al., 1990; Brandes et al., 1991; Coimbra et al., 1991; Barnes and Abboud, 1993; Khalil et al., 1993; Sporn and Roberts, 1993; Westergren-Thorsson et al., 1993; Yamamoto et al., 1993; Barcellos-Hoff et al., 1994; Kagami et al., 1994; Logan et al., 1994; Wolf et al., 1994; Wynn, 2007). However, very few studies have examined the direct role of TGF-β1 in skeletal muscle fibrosis (Fanbin et al., 2011). It was shown that TGF-β1 is expressed at high levels and is associated with fibrosis in the skeletal muscle of DMD patients (Yamazaki et al., 1994; Bernasconi et al., 1995; Zanotti et al., 2005; Cohn et al., 2007). Research has shown significantly higher levels of TGF-β1 mRNA levels in muscle biopsy specimens of patients with dermatomyositis (Confalonieri et al., 1997; Amemiya et al., 2000). The studies concluded that excessive TGF-β1 is correlated with chronic inflammation, the accumulation of ECM, and fibrosis (Confalonieri et al., 1997; Amemiya et al., 2000). Members of the TGF-β superfamily have been shown to be involved in Marfan syndrome and other inherited or acquired myopathies (Cohn et al., 2007; Burks and Cohn, 2011). We have used immunohistochemistry to study the expression of TGF-β1 and found strong expression of this cytokine in injured skeletal muscles in mice (Li et al., 2004). Taken together, these

results clearly implicate TGF-β1 in initiating a cascade of events that occur after muscle trauma or with the onset of muscle disease and suggest that neutralizing TGF-β1 or down regulating its expression could eliminate or reduce scar formation.

Modulating Fibrosis with Antifibrotic Agents

Our group recently showed that inhibiting the expression of TGF-β1 using several antifibrotic agents led to reduced muscle fibrosis. Muscle fibrosis is reduced resulting in nearly complete recovery of injured by using decorin, suramin, relaxin, gamma interferon (IFN-γ) and α-IFN (Fukushima et al., 2001; Chan et al., 2003; Foster et al., 2003; Sato et al., 2003; Chan et al., 2005; Li et al., 2005; Negishi et al., 2005; Cohn et al., 2007; Habashi et al., 2011). However, clinical use is hampered by lack of oral dosing formulations, serious side-effect profiles of some of these antifibrotic agents, and lack of U.S. Food and Drug Administration (FDA) approval for use in humans.

Research results had linked pathologic fibrosis in various tissues to an end-product of the blood pressure-regulating renin–angiotensin system, angiotensin II. Modulation of angiotensin II levels with angiotensin II receptor blockers or angiotensin converting enzyme (ACE) inhibitors has shown decreased fibrosis and improved function in kidney, liver, lung tissue, and the aortic wall (Lim et al., 2001; Paizis et al., 2001; Suga et al., 2002; Otsuka et al., 2004; Habashi et al., 2011). In diseases such as congestive heart failure, injured cardiac muscle is dysfunctional due to increased amounts of fibrosis. Use of ACE inhibitors or angiotensin receptor blockers to decrease the levels of angiotensin II in myocardium has demonstrated measurable improvement in cardiac output (Swedberg and Kjekshus, 1988; Gremmler et al., 2000; Habashi et al., 2011). Interestingly, investigators have observed a relationship between treatment for hypertension by the use of medications containing ACE and skeletal muscle health in the elderly. Patients treated with antihypertensive drugs had the unexpected positive side effect of decreased rates of muscle wasting and a reduction in the relative amount of adipose tissue within their musculature (Onder et al., 2006). In the other studies that utilized ACE inhibitors and studies of persons carrying a deletion of ACE gene, a direct effect of the renin–angiotensin system on the skeletal muscle was demonstrated (Folland et al., 2000; Onder et al., 2006). Muscle hypertrophic growth in response to overloading is believed to be modulated by the renin–angiotensin system (Folland et al., 2000; Onder et al., 2006). Other evidence has elucidated the mechanism by which the angiotensin II receptor blockade modulates TGF-β1 and showed that it is implicated in the prevention of muscle regeneration in murine models of Marfan syndrome (Cohn et al., 2007).

Angiotensin II receptor blocker, Losartan potassium (Cozaar; Merck & Co, West Point, PA), is an FDA approved drug that has minimal side effects, and has been in clinical use for over 20 years (Burks et al., 2011; Fakhfakh et al., 2011). Losartan use has been associated with reduction in fibrosis in several tissues (Lim et al., 2001; Paizis et al., 2001; Otsuka et al., 2004; Burks et al., 2011). Burks et al. (2011) have found that blocking angiotensin II Type I receptor by Losartan improved muscle remodeling in an aging mouse model, protected against sarcopenia by regulating TGF-β1 signaling pathways, and helped against muscle loss in immobilized hindlimbs by regulating the activity of the IGF-1/Akt/mammalian target of

rapamycin. Our group had investigated the effect of losartan on improving muscle healing after injury in a murine model and have found that losartan-treated mice exhibited a histological, dose-dependent improvement in muscle regeneration (Bedair et al., 2008). Furthermore, gastrocnemius muscles in mice given losartan solubilized in their drinking water and sacrificed 3 or 5 weeks after injury showed a significant reduction in fibrous tissue formation within the area of injury compared with control animals (Bedair et al., 2008). We are performing additional studies to understand the mechanism of action of losartan, as well as potential effects of dosing and timing of application to improve muscle healing in animal models (Kobayashi et al., in press).

The clinical implications for this application of angiotensin receptor blockers are potentially far-reaching and include not only sports and military-related injuries but also diseases like the muscular dystrophies. However, thus far there are no clinical reports on the use of this drug to optimize healing after muscle injury. We hypothesized that treatment of patients with doses of losartan that do not affect their blood pressure would antagonize the effects of TGF-β1, as we have shown in murine muscles (Bedair et al., 2008), and thus result in a clinically significant reduction of fibrosis formation after a common muscle injury. We have conducted a limited clinical case study on two college athletes to document the effect of losartan on muscle fibrosis and followed their tolerability and time needed to reach strength and flexibility levels that would correlate to ability to return to play. Furthermore, we followed up on any future recurrences of injury. Below we summarize the preliminary results obtained in this case study.

Case Study

At the initial evaluation in the clinic, a history and physical examination were performed on each of the two patient–athletes. The two patients had normal neurovascular examinations. They underwent a work up that included measurements of isometric hamstring muscle force and flexibility, plain radiographs of the pelvis, to rule out fracture of the ischial tuberosity, and magnetic resonance imaging (MRI; 1.5 T; GE-Sigma, Waukesha, WI) of the involved muscles was performed to better characterize the injury. The MRIs confirmed in both a partial thickness tear of the biceps femoris with surrounding edema. No fracture or significant hematoma was present. After obtaining the patients' consent for treatment, they were started on a 30-day course of losartan (Cozaar, Merck and Co.) at the oral daily dose of 50 mg. In addition to the medication, the subjects underwent routine standard of care rehabilitation supervised by a physical therapist. Return to sports activities progressed from jogging to running and sprinting. The patients were evaluated every 7 days by a study physician (Y.C.) that included measurement of blood pressure and hamstring flexibility and strength as described earlier. At the conclusion of treatment, both patients underwent testing on an isokinetic dynamometer (Biodex II; Shirley, New York) at 60° and 180° per second to assess the torque generating capacity of the hamstring and quadriceps muscles.

Serial isometric strength and flexibility measurements, performed at different time points, are summarized in Table 1. In addition to the isometric tests, iso-kinetic tests were performed. Both patients reported no side effects, while they were taking the losartan and remained normotensive throughout the 30-day course of the medication.

In both patients, we initiated treatment with losartan after the acute period of necrosis/degeneration and inflammation (10 days after injury for the first patient and 5 days after injury for the second patient). Patient 2 has demonstrated normal hamstring flexibility and strength by 3 weeks after injury and was without activity-limiting pain at that time (Fig. 4). Patient 1 was back to normal strength after 9 weeks. While meeting this criteria would normally correlate with appropriate time to return to athletics, both athletes suffered injury at the end of their season and thus a return to sport was not a primary end point. After 1 year, neither individual has suffered any recurrence of injury.

Extrapolating the time to return to play for hamstring injuries from the previous studies is a difficult and an inexact task, as evidenced by the many variables involved and a wide range of time reported in the studies ranging from mean of 14 days to 62 weeks (Pomeranz and Heidt, 1993; Slavotinek et al., 2002; Connell et al., 2004; Brooks et al., 2006; Askling et al., 2007, 2008). For our patients, both have been able to return to normal levels of activity after 9 weeks or even shorter for Patient 2. Despite the difficulty of extrapolating from this limited data, in our clinical experience this may be a shorter period of recovery than expected for patients with a similar degree of recurrent injury.

We are encouraged by the results of this case study that angiotensin receptor blockers may provide a clinically safe and effective option for a nonsurgical treatment of common skeletal muscle injuries. This may also be combined with growth factors and stem cell therapies. However, we do recognize the limitations of making treatment decisions based on case reports. We believe that a prospective, blinded, placebo controlled randomized clinical trial is necessary to determine the benefits of losartan use in comparison to conventional approaches, to the treatment of hamstring muscle rupture. Future research should consider the effects of the losartan on recovery of hamstring flexibility and strength, degree of fibrosis, time to return to full participation in sports, and the frequency of recurrence, as well as any adverse effects encountered by otherwise healthy individuals.

CONCLUSIONS

Review of recent literature indicates that muscle injury includes well-coordinated and interdependent phases, including degeneration, inflammation, regeneration, and fibrosis. Stem cells isolated from different tissues have great therapeutic potential, especially when combined with growth factors to modulate their growth and differentiation into certain lineages. From the skeletal muscle, our group has isolated a population termed MDSCs that has been effective in skeletal muscle repair and has great potential for future therapies for musculoskeletal diseases.

Biologically active signals produced by donor stem cells are believed to elicit response from donor cells and chemoattract host cells to the injury site. It is still unclear, though, which host cells are involved in the repair processes after stem cell transplantation. Blood vessel cells, immune and inflammatory cells, and resident cells at the injury site all appear to play a role in the regeneration process.

Recent investigations had shown that few donor stem cells are found within the regenerated tissues, but this should not detract from the potential of stem cells. Current findings indicate that the improvement in function is probably a result of paracrine signaling by donor stem cells (Yoon et al., 2005; Santhanam et al., 2007; Togel et al., 2007; Chen et al., 2008; Gharaibeh et al., 2011).

It is clear that the multipotentiality of the stem cells may not represent a major determinant for the success of stem cell therapy. This idea invites a revisit to the notion that using embryonic stem cells in therapeutic strategies is favored over adult derived stem cells because the latter are less able to differentiate into multiple lineages. We speculate that it is the stem cells' resistance to oxidative stress and paracrine signaling capacity that will lead to their increased ability to attract host cells, and it is this feature that is a key to successful stem cell therapy.

Choosing the most robust stem cell population(s) with the greatest potential for differentiation toward other cell lineages will continue to be of valuable interest to restore the structure and function of damaged tissues. Furthermore, additional efforts to isolate factors involved in the paracrine action of different stem cell populations under different conditions will very likely be utilized by future therapeutic applications.

The TGF-β1 is a cytokine that plays a significant role in the formation of fibrotic tissue in skeletal muscle and other tissues. Antifibrotic agents modulating TGF-B1 effects, including angiotensin receptor II blocker (such as losartan), are very effective in reducing muscle fibrosis and are likely to be a strategy that can be used in clinical therapies in the future. We believe that more rigorous clinical trials using losartan are necessary to determine the benefits of losartan use, in comparison to conventional approaches alone for the treatment of skeletal muscle injuries. This research should also consider the effects of the losartan on recovery of muscle flexibility and strength, and amount of fibrosis, as well as any adverse effects encountered by otherwise healthy individuals. Furthermore, future experiments on the use of losartan should compare the effects of using losartan on muscle healing when used with or without antiinflammatory drugs that seem to delay muscle regeneration.

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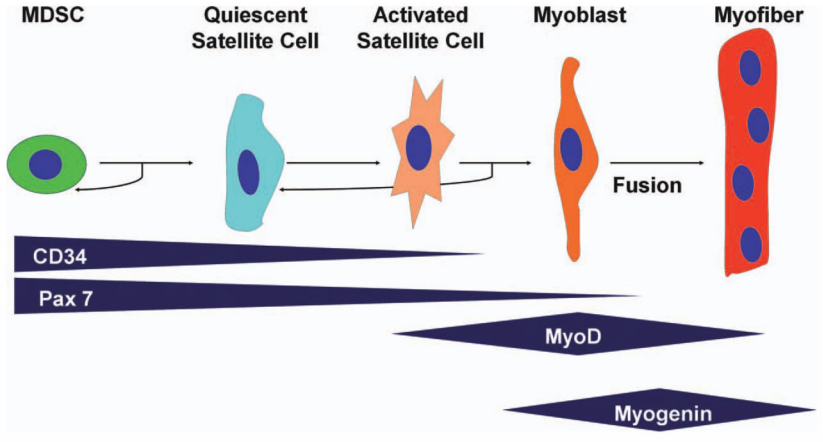

Figure 1.

Generalized scheme of myogenic differentiation. Other markers are used by different investigators. (Adapted from Deasy et al., 2001, Blood Cells Mol Dis, 27, 924–933)

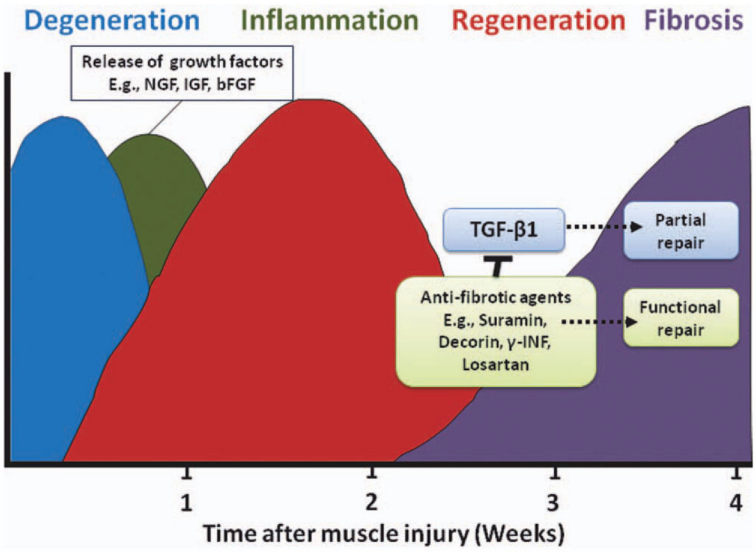

Figure 2.

Healing process in the skeletal muscle. Several overlapping phases are accompanied by the release of growth factors that modulate regeneration and formation of fibrotic tissue. Use of antifibrotic agents minimizes muscle scarring and leads to better functional outcome.

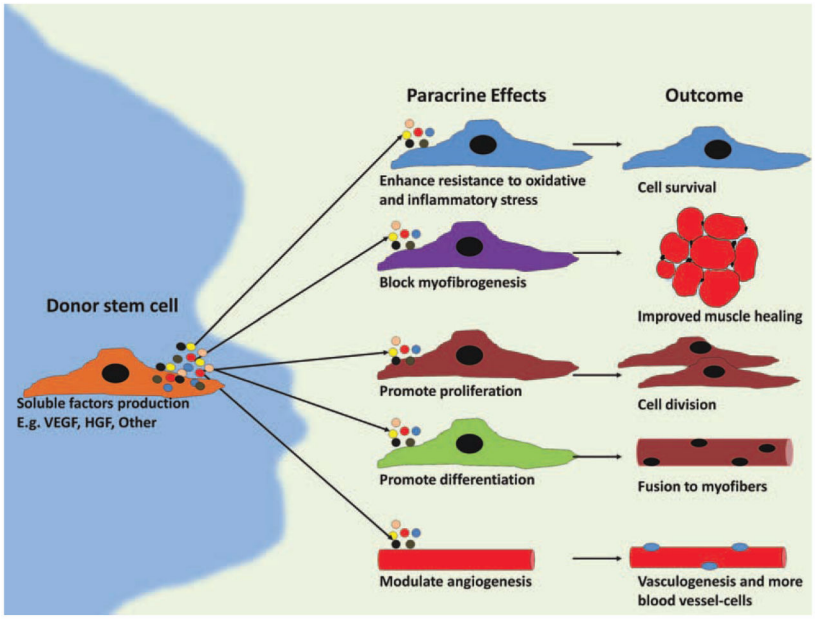

Figure 3.

Schematic of potential scenario of events taking place during cell therapy. Donor stem cells proliferate, differentiate, apoptose, senesce, or more importantly produce trophic factors that would have autocrine and paracrine effects on other donor and host cells. Furthermore, certain factors (e.g., VEGF) promote agniogenesis, which can positively affect other processes.

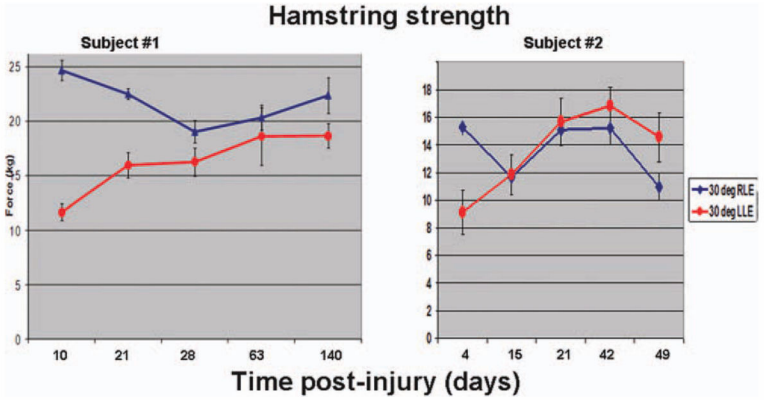

Figure 4.

Hamstring strength in two hamstring injury patients treated with Losartan (affected leg readings are charted in red).

TABLE 1

Results of Strength and Flexibility Measurements

	10 days postinjury		3 weeks postinjury		4 weeks postinjury		7 weeks postinjury		9 weeks postinjury	
	Strength (%)	**Pop angle (°)**	**Strength (%)**	**Pop angle (°)**	**Strength (%)**	**Pop angle (°)**	**Strength (%)**	**Pop angle (°)**	**Strength (%)**	**Pop angle (°)**
Subject 1	47.2/54.1	30 (5)	71.0/79.6	5 (5)	85.5/92.6	5 (5)	91.5/83.5	5 (5)	83.3/107.1	5 (5)
Subject 2	59.6/60.1	13 (10)	101.4/85.5	24 (15)	103.8/92.5	15 (12)	110.5/99.7	26 (18)	132.5/110.8	14 (12)

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