Enhancing Myoblast Proliferation by Using Myogenic Factors: A Promising Approach for Improving Fiber Regeneration in Sport Medicine and Skeletal Muscle Diseases

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Abstract

Macrophages drive muscle regeneration and repair by removing necrotic material and producing key signaling molecules. The array of cytokines/growth factors produced by macrophages and myogenic cells stimulates the proliferation, migration and differentiation of satellite cells. Although the details of such processes are only partially understood, it is known that the administration of purified growth factors can improve the final outcome after traumatic muscle injuries. Also, such approach has proved to be beneficial in myoblast transplantation experiments in animal models. The translation of such procedures into therapeutic protocols is, however, hampered by high costs and the somewhat oversimplified biochemical input compared to the physiological signal network.

We have previously reported that peritoneal macrophages could secrete factors capable of increasing the myoblast/myotube yield in cultures of primary rat myoblasts. Recently, we observed that a macrophage cell line could be stimulated to produce a conditioned medium that specifically enhances the proliferation of cultured neonatal primary myoblasts from mouse, rat, chicken, and human fetal myoblasts. The factors did not inhibit differentiation and led to a striking increase in the rate of contractile myotube formation. The factors could also enhance muscle regenerative processes *in vivo*, thereby suggesting a potential role as an economical and effective tool for the treatment of traumatic and disease-related muscle injuries. Further experiments in this direction and the biochemical characterization of the macrophage-produced myogenic factors are presently underway. The possibility to use the macrophage factors to improve the myoblast yield from diseased-muscle biopsies is also under investigation.

Abbreviations: MCM: macrophage-conditioned medium.

Keywords: macrophages, cytokines, muscle regeneration, myoblast proliferation, myoblast transplantation

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Introductory considerations

Mature skeletal muscle is able to respond to a variety of injuries (mechanical, chemical or biological) by forming new functional fibers thanks to the activation of its population of satellite cells located between the sarcolemma and the basal lamina of mature myofibers. These are quiescent cells that start to proliferate in response to muscle damage, generating a large number of myoblasts that then fuse together to form myotubes and new myofibers [1]. The same process takes place in the early phases of Duchenne muscular dystrophy, where the continuous cycles of muscle degeneration-regeneration eventually lead to the depletion of the physiological reservoir of satellite cells and to fibrous-fat tissue substitution [11]. In contrast, a healthy individual has the potential to successfully repair both chronic and acute muscle damage (derived, for example, from intense physical activity). Still, the time course and the final outcome of the repair processes (e.g. the prevalence of new myofibers versus fibrotic tissue) can widely vary, depending on the individual's specific physiology and the type and severity of injury. In any case, even in healthy individuals the muscle repair processes become less efficient with aging.

Innovative biotechnological tools, aimed at improving fiber regeneration in sport medicine and skeletal muscle

diseases, are presently the subject of great interest. For this reason, many laboratories have set out to further investigate which growth factors are involved in the natural healing process and have directly tested them as therapeutic tools. So far, two main strategies have been conceived: (a) the direct *in vivo* administration of the factors into damaged muscle areas and (b) the use of growth factors to increase the number of *ex-vivo* transplantable muscle progenitors.

The main growth factors that are known to play a role in myoblast proliferation and/or differentiation are listed in Table 1; at the molecular level some of the key intracellular events that follow their binding to the corresponding receptors have previously been described [39]. There are now a number of studies that have demonstrated how some of these factors can be used to improve muscle healing *in vivo*. Such approaches are, however, still hampered by the fact that using high concentrations of a single pure factor represents a rough over-simplification of the complex spatial-temporal network of signals involved in muscle regeneration. In some cases, such limitation could even lead to an impairment of the normal healing physiology, despite the *in vitro* promising effect. Indeed, some *in vivo* results were not always reproducible amongst different reports (see below).

In this context, we developed a fast and economical system to produce large amounts of macrophage-released myogenic factors that, in principle, could more closely mimic the 'cocktail' of signaling molecules involved in natural muscle repair (Fig. 1). We are presently assaying their effect on myoblast proliferation and differentiation, both *in vitro* and *in vivo*.

The use of growth factors to improve muscle recovery after traumatic injuries. The crucial role of IGF-I.

Muscle injuries are involved in up to 50% of sport-related accidents [25] and can be classified as shearing injuries (when both the fibers and the framework have been affected) and *in situ* injuries (when only the fibers have been damaged). In both cases, the healing process follows the same multi-stage pathway. Immediately after the damage, the necrosis and degeneration of the damaged tissue triggers the acute inflammatory

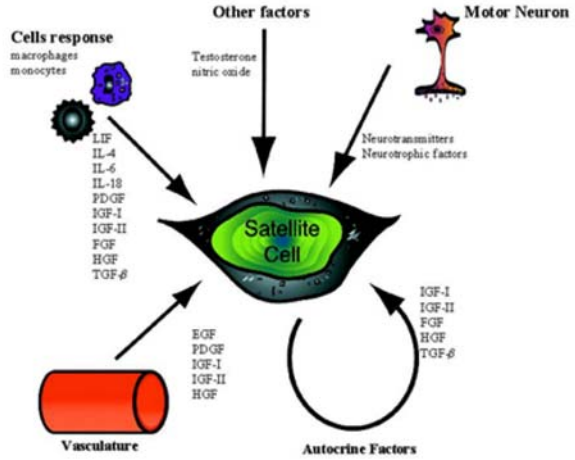

Figure 1. Schematic representation of signalling molecules secreted by several cell lines during muscle regeneration. (Picture modified from Hawke, 2001).

response. The macrophage-driven phagocytosis of the tissue debris is then followed by the activation of satellite cells, leading to the formation of large amounts of proliferating myoblasts which then fuse to form the precursors of the new fibers, the myotubes. Of course, the formation of a new capillary bed is also required. Finally, the regenerating fibers are re-innervated and the newly formed tissue recovers its full contractile functionality. It is important to note that in the case of shearing injuries, the repair process does not only involve the muscular tissue, but also the connective-fibrous components. Overall, the repair process is very efficient and can lead to a complete recovery. However, in the presence of extensive injury and/or of a physiologic deficit the concurrent formation of connective scar tissue can overcome the actual fiber regeneration, thereby preventing a complete functional restoration [21].

Recently, Menetrey and colleagues reported that intramuscular administration of insulin-like growth factor type I (IGF-I) can improve the outcome of muscle regeneration in a murine model of traumatically damaged skeletal muscle [28]. The mechanism of action

Factors	Biological effects on myofiber formation
IGF-I	Stimulation of proliferation and muscle differentiation
IGF-II	Stimulation of proliferation and muscle differentiation
HGF	Stimulation of proliferation and inhibition of differentiation
FGF	Stimulation of proliferation and inhibition of differentiation
TGF-β	Stimulation of proliferation and inhibition of differentiation
IL-6	Stimulation of proliferation
LIF	Stimulation of proliferation

Table 1. Factors that regulate myofibers formation during muscle development and regeneration

appears to involve the stimulation of myoblast proliferation, which then leads to a higher number of regenerating fibers one week after injury (more than three-fold compared to controls) and to better contractile properties after one month (fast twitch and tetanus strength were twice as high in treated animals). A critical role of IGF-I, at least partially shared with IGF-II, during the (re)generation of the skeletal muscle also relies on its effect on myotube hypertrophy, as measured by post-mitotic increases in diameter, total protein, and the number of nuclei within the myofibres. IGF-I increases satellite cell proliferation, gene transcription, and protein translation. and inhibits muscle proteolytic activities: each of these processes contributing to muscle hypertrophy [18]. Indeed, transgenic mice expressing IGF-I in their skeletal muscles showed an increase in muscle mass and strength and retained the ability to proliferate in response to damage even in elderly individuals [32].

Even though IGF-I protein exists in multiple isoforms, most of the data dealing with skeletal muscle have focused on the IGF-IEa isoform [12]. However, Hameed et al. (2003) found no significant increase in IGF-IEa expression in either young or old human individuals after resistance exercise training [17]. In contrast, the IGF-IEb isoform (termed mechano-growth factor, as it only appears to be produced by damaged or loaded skeletal muscle) increased after resistance exercise in young adults but not in elderly subjects. When stimulated with exogenous IGF-I, satellite cells isolated from aged animals maintain their proliferative capacity [6], suggesting that age *per se* is not necessarily a limiting factor in muscle hypertrophy or repair. In conclusion, it is possible that during aging the *in vivo* stimuli for increased satellite proliferation became limiting, IGF-IEb being one of the 'missing' growth factors. Indeed, the *in vivo* application of IGF-I restored satellite cell proliferative potential in immobilized old skeletal muscle and rescued about 50% of the lost muscle mass [6].

IGFs could also be essential in ensuring the correct sequence of events in the repair of injured muscles. In fact, IGF-I and II exert a biphasic action on skeletal muscle, as they first increase cell cycle markers and cell proliferation and then allow the activation of myogenic transcription factors, structural skeletal muscle gene expression, and myotube formation [22,36]. In mice, not only IGF-I but also basic fibroblast growth factor (β-FGF) and nerve growth factor (NGF) could enhance muscle regeneration in lacerated, contused or strain-injured muscle when directly administered into the affected areas [21 and references therein]. However, it should be mentioned that the good regenerative activity reported for β-FGF appears to be in contrast with previously reported results [30].

As mentioned above, during muscle regeneration subsequent to a severe injury there is a competition between the formation of the connective scar tissue and new functional myofibers. Individual growth factors can improve muscle regeneration but cannot prevent muscle fibrosis. Indeed, myoblast-mediated IGF-I gene transfer was able to improve muscle healing but the functional recovery was still incomplete. Two reasons are evoked to explain this situation: (a) IGF-I-mediated fibroblast proliferation and deposition of extracellular matrix, and, the endogenous secretion of fibrotic factors can interfere with the myogenic activities of IGF-I itself after injury [23]; (b) other late factors secreted after injury continue to stimulate scar tissue formation even after muscle self-repair [21]. These possibilities are supported by the observation that direct *in situ* administration of TGF-ß blocking agents (such as Decorin and Suramin) was able to improve the final outcome of muscle repair. High levels of TGF-β1 expression are associated with fibrosis, accumulation of extracellular matrix, and chronic inflammation in Duchenne dystrophy and dermatomyositis. Suramin inhibits TGF-β1 expression by competitively binding to its receptor. *In vivo*, direct injection of suramin (2.5 mg) into injured murine muscle resulted in effective inhibition of muscle fibrosis and enhanced muscle regeneration and functional recovery [7, 13].

A correct balance between inflammation and regeneration appears to be an essential pre-requisite for successful muscle repair. NF-κB is a major transcription factor that modulates the cellular immune, inflammatory, and proliferative responses and has been shown to participate in the IGF-I dependent intracellular cascade events in myogenic cells, although in a transient fashion [37]. The constitutive activation of the NF-κB pathway has been demonstrated in Duchenne muscular dystrophy and inflammatory myopathies [31]. The systemic administration of curcumin, a pharmacological inhibitor of the NF-kB known for its anti-inflammatory properties, greatly enhanced the kinetics and extent of murine muscle regeneration *in vivo*, following freeze-induced fiber necrosis [37].

Taken together, these observations highlight the potential clinical use of IGF-I for the treatment of muscle dysfunction associated with neurodegenerative diseases, injury or aging.

The role of macrophages in muscle repair and plasticity

As mentioned above, early muscle repair is accompanied by an inflammatory response in which macrophages remove the cellular debris derived from the necrotic fibers [18, 22, 35]. Macrophages, however, are also known to produce a variety of cytokines responsible for mitosis regulation and chemotaxis (such as FGFs, FGF-ß, PDGF, LIF, IL-6, IL-10, IL-12, TNF-α) and it is now recognized that they play a complex role in the plasticity of skeletal muscle [see for example 35, 22, 18]. McLennan has reported that resident macrophages do not have any phagocytic role during

muscle regeneration but rather secrete chemotactic molecules that attract blood-borne macrophages to the injured site [27]. We have shown that co-cultivation of rat or human primary myoblasts with macrophages from peritoneal exudate or peripheral blood monocytes, respectively, resulted in a substantial increase of myogenic cell proliferation and myotube formation [2, 3]. Similar proliferative results have been reported in primary myoblasts derived from mouse and turkey [29], even though in this latter case the authors also reported an inhibitory effect on myotube formation.

The existence of a regulatory role of macrophages in muscle regeneration has also been demonstrated *in vivo*. Lescaudron and colleagues have in fact shown that blood-borne macrophages are essential for the successful graft of transplanted muscle and that pre-treatment of the graft before implantation with macrophage inflammatory protein 1-ß leads to a significant increase in myoblast proliferation [26]. Another series of results indicate that some macrophage secreted cytokines, amongst which are Leukemia Inhibitory Factor (LIF) and FGF-ß, can be used to enhance muscle regeneration and myoblast grafting [38 and references therein].

The effects of LIF have also been investigated by Kurek and colleagues, who reported that muscle regeneration was significantly hampered in a LIF knockout mouse. Most notably, the targeted infusion of LIF was able to correct the mutant phenotype [16]. Another macrophage-produced cytokine that is known to play a role in muscle regeneration is IL-6, which shares a receptor component with LIF. In particular, IL-6 increases the degradation of necrotic tissue and stimulates macrophage apoptosis [4]. IL-6 also synchronizes the cell cycles, but does not stimulate proliferation of satellite cells [reviewed in 18].

Other studies have reported that inflammatory cytokines, known to be secreted by macrophages (LFA-1 and IL-1) as well, were at least partially responsible for the massive death of transplanted myoblasts that many research groups detected within the first 24-48 hours after injection [15; 34]. In general, when considering the role of cytokines/growth factors in muscle regeneration and repair, one should keep in mind that both hematopoietic and myogenic (myofibers and myoblasts) cells are an important source of signaling molecules, which play a key role in the process (e.g., IGF-1 and IGF-II, see [18] and Fig.1 for a more comprehensive description of the myogenic signalling network). Interestingly, a recent report indicated that myoblast fusion into pre-existing myotubes is promoted by myotube-secreted IL-4, the same cytokine responsible for the regulation of the fusion between macrophages, thus suggesting a conserved mechanism for cellular fusion [20]. This is the first example of IL-4 production by cells that are not part of the immune system. Such observation has prompted the speculation that the involvement of marrow-derived hematopoietic cells, which has been documented to occur during muscle regeneration [24], might actually be a consequence of illegitimate fusion between macrophages and growing myotubes [8].

Production of myogenic factors from an established macrophage cell line

The initial experiments we carried out to identify the nature of the macrophage-produced myogenic factors used peritoneal macrophages as starting material and suggested the active component(s) was a water-soluble protein of 10-50 kD. Such approach, however, is severely hampered by two problems: the low abundance of the factor(s) in a complex mixture that contained very high levels of serum proteins and the variability between different preparations of peritoneal macrophages [10, 14].

More recently, we have found that a murine macrophage cell line could also be induced to release muscle-specific growth factors when cultured in serum-free medium. Supplementing primary myoblast cultures derived from neonatal rat with 20% v/v of such macrophage-conditioned medium (MCM) almost doubles the percentage of proliferating MyoD-positive cells and slightly delays myoblasts differentiation (i.e., there is a decrease in the percentage of myogenin-positive cells until total confluence is reached). It is important to note, however, that once the myoblasts expanded in the presence of MCM have reached 100% confluence and are switched to fusion (low-serum) conditions, the extent of myotube formation remains the same, both in the presence and in the absence of the conditioned medium. Importantly, the presence of MCM does not have any influence on the proliferation rate of MyoD-negative cells [5].

The effect of MCM is even more striking when tested on cultures of primary rat myoblasts, that also include a large proportion of non-myogenic cells. Under these conditions, there is little or no myoblast fusion even after 10 days in the differentiation medium (Figure 2, upper panel), whereas the same cells grown in the presence of MCM show the formation of an impressive network of myotubes that are capable of contracting for an extended period of time (Figure 2, lower panel).

The effect of MCM has also been tested *in vivo*, in a rat model of muscle regeneration induced by extensive tissue ablation. In these experiments, approximately 80% of the muscle mass was surgically removed from the Tibialis Anterior muscles of young rats and MCM was injected 24 and 48 hours after surgery. The immunohistochemistry analysis showed that both seven and fourteen days after surgery the muscles that received the MCM injection displayed a substantial increase in the number and size of regenerating fibers compared to non-injected controls. Also, two weeks after surgery the treated muscles had recovered a double

amount of tissue mass compared to sham-injected controls [5].

Future directions for MCM characterization

These initial and encouraging results obtained with MCM will be probed further using a new series of experiments, both *in vitro* and *in vivo*.

In the former case, we plan to investigate the possible effect of MCM stimulation on the production of primary myoblasts derived from human bioptic material. We want to verify if the striking proliferative effect that we found in cultures derived from neonatal or fetal muscle can also be reproduced with myogenic cells derived from adult human muscle biopsies of healthy, dystrophic, and elderly donors. Should this be the case, MCM treatment could be of great value for improving myoblast transplantation protocols in dystrophic patients. In such approaches, in fact, there is always a need for a large number of cells for the transplant, which means that a significant amount of muscle tissue should be used as a source of myogenic cells. Such a problem is obviously much more relevant when planning to use *ex vivo*-transfected autologous cells from dystrophic patients, whose reservoir of satellite cells has already been depleted. Therefore, any procedure that could increase the number of myogenic cells obtainable from bioptic tissue would be greatly beneficial. In addition, the same approach might favor the studies and future applications related to the aging effect on muscle regenerative capacity.

As for the *in vivo* studies, we are presently repeating the experiments of muscle reconstitution in rats using a greatly improved protocol, which includes the replacement of the empty volume left by the removed muscle with a biodegradable organic matrix. This variation will also more closely reproduce the procedures used for plastic surgery in humans, in which the use of biocompatible fillers for tissue reconstitution is becoming more and more common. We believe that this change will improve the reproducibility of the MCM-induced regeneration performance over what was found in the preliminary studies [5]. The presence of the matrix will, in fact, provide a scaffold for the growth of proliferating cells and also allow a better persistence of the injected factors in the affected areas.

At the same time we are also testing the effect of MCM on experimental models that reproduce the different, and less severe, muscle injuries that would be more relevant to sport medicine. For this purpose, we will administer MCM into well-defined shearing injuries created within muscles of adult rats, as well as into murine muscles after they have suffered exercise-induced damage. In both cases, we will compare our results with those reported in the literature for the administration of growth factors, as described in the previous sections.

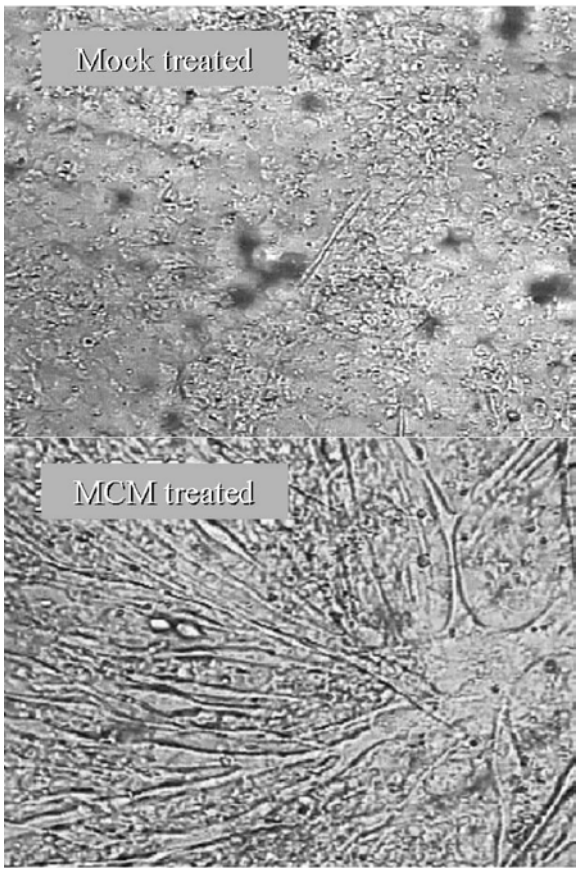

Figure 2. Effect of Macrophage-Conditioned Medium (MCM) in rat primary cell culture enriched with non-muscle cells. The images are captured after 10 days. In these conditions, only very small and extremely rare myotubes can be observed on mock-treated plates: one is showed in the central part of the field. In contrast, MCM induces the formation of a large network of myotubes able to contract for a long time.

Should these series of experiments yield positive results, MCM will prove to be beyond a doubt a valuable source, easy and cheap to produce in large scale, of muscle-specific factors that could be useful in the treatment of traumatic or disease-induced muscle injuries.

We have also started a series of experiments aimed at the detailed biochemical characterization of the active components of MCM. This information is not only fundamental in the view of any possible therapeutic applications, but, in our opinion, will also provide important insights into the complex biology of the macrophage-myoblast interplay during muscle repair and regeneration.

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