Control of Cell Proliferation by Macrophage-Myoblast Interactions

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Abstract

After injury muscle fibers undergo a process of degeneration, followed by regeneration of the damaged fibers. The damage-induced inflammation is characterized by late macrophage infiltration and activation of muscle precursor cells, i.e., satellite cells. In a period of several days, proliferation and fusion of satellite cell-derived myoblasts form new fibers. In view of the emerging importance of myofiber regeneration in the management of muscle diseases and gene therapy, we have been investigating the pivotal role played by macrophages in myoblast proliferation. We previously showed that rat and human macrophages, besides their scavenger role, selectively induce myoblast proliferation by releasing soluble factors. Evidences were collected in proliferating and differentiated primary muscle cultures by measuring two markers of muscle cells, i.e., desmin and myosin heavy chain. Though we still have not identified the macrophagic cytokine(s) which enhances myoblast proliferation *in vitro*, we have evidence that cytokines are also secreted by human myoblasts. ELISA quantitation shows that in muscle culture the concentrations of TNF, IL-1 and IL-6 is significantly higher than those present in the medium. The autocrine secretion of IL-6 is particularly high and its concentration is related to myoblast proliferation. When macrophage-conditioned-medium is deprived by immunochromatography of IL-6 and LIF (both of them are erythropoietin-like cytokine) the myoblast-proliferation effect is not removed, thus the macrophage produced soluble factor is not IL-6. In macrophage-myoblast coculture over time there is both an increase of myoblast proliferation and a decrease of macrophage content. Recent report of Transferase UTP Nick End Labeling (TUNEL) positive macrophages in regenerating muscle suggests a role of apoptosis is resolution of muscle damage-induced inflammation. Data we are collecting show that addition of exogenous IL-6 induces an impressive burst of apoptosis in cultured monocytes. Taken together, all these findings open a new perspectives on the reciprocal influences of macrophages and myoblasts and suggest that IL-6 may act as a secondary signal promoting myoblast proliferation and fusion, and as a regulatory retrograde factor for resolution of inflammation by apoptosis of infiltrating macrophages.

Key words: human satellite cell, myoblast proliferation, IL-6, cytokines, macrophage, activated monocyte, apoptosis.

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Skeletal muscle fibers result from the fusion of embryonic myoblasts into myotubes during the early stages of development. The myotubes then synthesize contractile proteins characteristic of mature muscle fibers. Some myoblasts, however, do not undergo fusion but remain quiescent (satellite cells), located between the sarcolemma and basal lamina of mature myofibers. After muscle injury, the muscle fibers degenerate and there is an inflammatory reaction which is accompanied by satellite cell activation and production of new myoblasts. In a few days, fusion of the newly generated myoblasts forms new fibers.

After injury muscle fibers undergo a process of degeneration, followed by regeneration of the damaged fibers. Beside myofiber damage, one of the earliest visible cellular events in the skeletal muscle regeneration is the accumulation of polymorphonuclear leukocytes at the site of damage. Two days later, macrophages infiltrate and phagocytise the necrotic muscle. Afterward, myoblasts proliferate, fuse and regenerate the lost myofibers [2].

We demonstrated that both in satellite cells and primary myoblast cultures of rat, the presence of macrophages is able to orchestrate the mitotic activity of muscle cell pre-

cursors by secreting soluble growth factors [6, 7, 9]. A myriad of growth factors and cytokines have been shown to affect proliferation and differentiation of myogenic cells in culture [3, 15, 16, 18, 27], and many of them are secreted by macrophages [21, 26]. Macrophage and satellite cell co-cultures show a significantly increased number of differentiated myotubes when compared to control cultures of satellite cells alone. Additional experiments with macrophage-conditioned media revealed that the increase in muscle cell proliferation is mediated by acid-stable, heat-labile soluble factor(s). These results suggest that besides their scavenger role, macrophages influence rat myoblast proliferation through one or more soluble factors they release [6, 7, 9]. Of these, leukemia inhibitory factor (LIF), interleukin-6 (IL-6), and a few others have been considered good candidates, since they belong to the erythropoietin family of mitogenic cytokines [21].

By using human satellite cell- and peripheral monocyte-derived co-cultures we have extended our previous observations to man [10]. Furthermore, an ELISA quantitation showed that the myoblast secretion of IL-6 [4] is increased and associated with increased proliferation of human satellite cells supplied with activated monocyte-conditioned medium (MCM) [10]. We have now new data showing that when IL-6 and LIF are subtracted from MCM by immunochromatography, the increased myoblast proliferation does not cease. These results suggest that a growth factor other than either IL-6 or LIF is secreted by macrophages and stimulates both proliferation and IL-6 production by myoblasts which in turn causes the increased rate of myoblast proliferation.

A short summary of the methods we used follows. Human monocytes harvested from peripheral blood were activated *in vitro* by heterologous IgG or lipopolysaccharide (LPS) [20]. Activated monocyte-conditioned medium harvested at day three of culture was added to myoblast cultures as described in [10]. Myoblasts derived from human satellite cells were obtained from biopsies of adult patients during toeletting of previous aesthetic surgery. Control cultures, seeded at 8,000 myoblasts per cm^2, were grown in 10% FCS and 0.2% AB type human serum [24]. Three doses of 10% activated monocyte-conditioned medium were added to human myoblasts during the first week of culture. Quantitation of muscle growth was determined by SDS PAGE and immunoblotting of desmin content in muscle culture extracts [6, 7, 29, 34, 35]. IL-6 was measured in culture supernatants by ELISA (Genzyme). These measurements were taken on the supernatants from 3-day human muscle cultures, which either had five additions of IL-6-deprived-MCM or of standard MCM as controls. IL-6 and LIF were removed from MCM by immunochromatografy (Dalla Libera et al., manuscript in preparation). Myonuclei counting was performed as follows: Six different microscopic fields for each well were photographed. Myouclei were identified after propidium iodide staining of desmin- or embryonic myosin-positive cells. Cell cultures were stained with anti-desmin (Boeringher) and anti-embryonic myosin heavy chain (generous gift of Professor S. Schiaffino, Padova, Italy) monoclonal antibodies as previously described [6]. In some rat experiments myonuclei were identified by anti-MyoD monoclonal staining. DNA fragmentation in nuclei of macrophages was visualized by the Transferase UTP Nick End Labeling (TUNEL) method (Apoptag kit, Oncor, Gaithersburg, MD) and DNA laddering [28, 32].

Early myotubes appear one week after plating in our human control cultures derived from satellite cells. After an additional week large cross-striated multinucleated myotubes are present; they stain strongly with anti-embryonic myosin heavy chain antibodies. When human myoblasts are grown in the presence of either activated monocytes or of supernatant from activated monocyte cultures, myotubes are even more numerous and contain more nuclei [10]. Increased growth of human myoblast/myotubes was confirmed by measuring the amount of desmin in the cultures by SDS PAGE separation and immunochemical analysis. In human muscle cultures supplemented with activated monocyte medium the amount of desmin is 3 to 10 fold higher than in controls, thus demonstrating that myoblast proliferation is enhanced. We previously showed that the amount of desmin and myosin found in rat muscle cultures correlates well with the extent of proliferation and differentiation the cells have undergone [6, 7]. Recently we confirmed these results by counting myonuclei after staining with anti-MyoD antibodies, a very specific marker of skeletal muscle commitment (Cantini et al. manuscript in preparation).

The current model of myogenic differentiation, derived largely from studies of myogenic cell lines, is essentially a negative regulation model in which mitogenic growth factors (e.g. FGF, PDGF and TGF) inhibit the differentiation process [23]. Therefore, cytokines and growth factors can stimulate either myoblast proliferation or differentiation [15, 16, 18, 27]. Many of them are secreted by macrophages [26]. In particular, IL-6 and LIF have been shown to increase human myoblast proliferation [3]. The production of IL-6, a pleiotropic cytokine not usually produced by normal cells, is induced *in vitro* in many cell types by a variety of stimuli. Human myoblasts constitutively secrete IL-6 *in vitro* , and added TNFα has been shown to have the potential to greatly increase its secretion [4].

Measuring by ELISA IL-6 concentration in the culture media, we confirm that macrophages secrete specific factor(s) which increase myoblast proliferation and IL-6 production [10]. IL-6 content in the growth medium is low in comparison to that of the control muscle culture medium. After addition of activated monocyte-conditioned medium, IL-6 concentration in muscle culture medium increased more than five times in comparison to control cultures. The activated monocytes also secrete IL-6, but the amounts of monocyte-conditioned medium added to muscle cultures cannot account for the very high levels of IL-6 present in muscle cultures. Since IL-6 appears to be constitutively secreted by myoblasts, one can say that the

higher the IL-6, the more enhanced the cell proliferation or the more enhanced the cell proliferation, the higher the IL-6 myoblast secretion.

To confirm the hypothesis of the mitogenic role of IL-6 for myoblasts, MCM was deprived of IL-6 and LIF by immunochromatography. The treatment drastically reduces IL-6 in MCM. On the other hand, the IL-6 and LIF deprived MCM is able to both increase IL-6 autocrine production and proliferation of human myoblasts *in vitro*, as the untreated control MCM, suggesting that the mitogenic activity of MCM is not due to its IL-6 content (Cantini et al. manuscript submitted).

The actual number of cells in culture depends on the relative rate of cell proliferation, differentiation and death. In particular, it is now emerging that both *in vitro* and *in vivo* myoblasts, myotubes and myofibres beside necrosis undergo to apoptosis [11-14, 25, 28, 31-33, 36-39, 42]. Therefore we counted apoptotic cells in our experimental conditions, and we were surprised to find that macrophages, not myoblasts increase apoptotic process when grown in the presence of IL-6. These preliminary results may explain our previous observation that in cocultures muscle cells increase in number, while macrophages almost disappear. There are emerging evidence that regulation of monocyte apoptosis may by pivotal in the outcome of an inflammatory response, and inflammatory cytokines can either promote or prevent monocyte apoptosis. It has been demonstrated that human peripheral blood monocytes die by an apoptotic process when cultured in the absence of growth stimuli, but that this process is effectively reversed by addition of inflammatory mediators such as LPS, IL-1β or TNF-α. Moreover , this activation-dependent survival of monocytes is abolished by IL-4, an anti-inflammatory cytokine, suggesting that apoptosis may represent the mechanism for eliminating activated monocytes that become no longer necessary as inflammation wanes. A recent report shows apoptosis of macrophages during resolution of muscle inflammation in unloaded-reloaded rat muscles [40]. Either IL-6 act as a primary factor or its action is mediated by other cytokines known to induce apoptosis of macrophage *in vitro* [1, 30], remain to be determined. The observation that Fas mediates apoptosis in monocytes by a reactive oxygen intermediate dependent pathway emphasizes the complex network of signals that control the life span of monocytes [41]. Anyhow, if one take into account the role of polymorphonuclear leucocytes in satellite cell activation and chemotaxis [16, 18], and that cytokine-induced apoptosis of polymorphonuclears is a signal for their phagocytosis by macrophages in early phases of ceasing inflammation [17], all our findings open a new perspectives on the reciprocal influences of macrophages and myoblasts and suggest that IL-6 may act as a secondary signal promoting myoblast proliferation and fusion, and as a regulatory retrograde factor for resolution of inflammation by apoptosis of infiltrating macrophages in regenerating muscle.

Therefore we suggest that cytokines released by myoblasts not only act as proliferating and/or differentiating agents, but influence macrophage activity, in particular acting as suppressor of inflammatory events through their potential role as inducers of apoptosis of macrophages. Figure 1 summarizes our actual views on the reciprocal influences of macrophages and myoblasts in muscle regeneration. These hypotheses are particularly interesting, since new applications of gene therapy via myoblasts could be open by a recent report of prevention of allograft rejection with engineered myoblasts expressing FasL [22]; indeed, immunological rejection of allografts remains a major obstacle toward the application of engineered cell transplant in gene therapy. Interactions of Fas with its ligand (FasL) are thought to play a major role in the maintenance of immunological homeostasis and peripheral tolerance [43]. Provision of the FasL signal by transfected syngeneic myoblasts within the local environment of a cell allograft could deliver a death signal to infiltrating allo-activated T cells that express Fas, and thereby protect the allograft from rejection. This approach could permit the use of plasmid-based gene delivery into myoblast carrier cells and make unnecessary the direct transfection of allograft cells in many different applications, such as pancreatic islet allograft for the treatment of diabetics. The results of mid-term experiments are very promising [22], and therefore open new perspectives to our studies of macrophage-myobalst interactions. Cytokines released by both macrophages and myoblasts could be essential for myoblast proliferation and fusion, and for resolution of inflammatory reactions during muscle regeneration. It remains to be determined whether known or unknown cytokines are responsible for the selective proliferation of myoblasts and if they could have a role as a growth factor regulator of satellite cell activities in meat animals [18], in

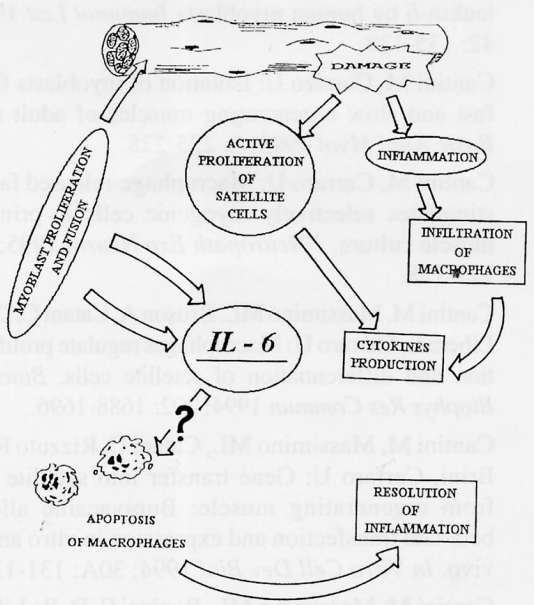

Figure 1. Reciprocal influences of macrophages and myoblasts in muscle regeneration.

control of pathogenesis of exercise-induced muscle damage [12, 42], in gene therapy via engineered myoblasts in muscle dystrophy (one of the problem there is the immunological response induced by engineered myoblasts [5, 8, 19]), and in myoblast-allograft cell transplants in many other different applications [22].

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