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M. Cantini • E. Giurisato • C. Radu • S. Tiozzo • F. Pampinella
D. Senigaglia • G. Zaniolo • F. Mazzoleni • L. Vitiello

Macrophage-secreted myogenic factors: a promising tool for greatly enhancing the proliferative capacity of myoblasts in vitro and in vivo

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Abstract In this work we set out to determine if the murine macrophage J774 cell line can be used to produce myogenic growth factors. Activated J774 macrophages were grown in serum-free conditions. The macrophage-conditioned medium (MCM) was then used to treat cultures of primary myoblasts and regenerating muscle tissue, in vitro and in vivo respectively. MCM activity in vitro was tested by analyzing the expression of muscle-specific transcription factors, in parallel with the proliferation and differentiation rates of the cells. The macrophage-secreted factors greatly enhanced the proliferative potential of both rat and human primary myoblasts and were found to be highly muscle-specific. In vivo, MCM administration markedly enhanced the regenerative processes in damaged muscles. The ability to produce large amounts of macrophage-secreted myogenic factor(s) in the absence of serum holds great promise for its biochemical characterization and successive application in therapeutic protocols, both for ex vivo gene therapy and for muscle repair.

Key words Macrophages • Cytokines • Muscle regeneration • Myoblast transplantation

M. Cantini (✉) • E. Giurisato • C. Radu • S. Tiozzo • F. Pampinella
D. Senigaglia • G. Zaniolo • F. Mazzoleni • L. Vitiello
Department of Experimental Biomedical Sciences
University of Padua, Via Bassi 58/B, I-35121 Padua, Italy

Introduction

Mature skeletal muscle is able to respond to a variety of injuries (mechanical, chemical or biological) by forming new functional fibers through activation of its population of satellite cells. 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, eventually, new myofibers [1, 2]. The repair process is accompanied, during its early stages, by an inflammatory response in which macrophages remove the cellular debris derived from the necrotic fibers [3–5]. Macrophages, however, are also known to produce a variety of cytokines responsible for mitosis activation and chemotaxis [5–7]. In our laboratory we have previously shown that co-cultivation of rat or human primary myoblasts with macrophages obtained from a rat peritoneal exudate or peripheral blood monocytes, respectively, resulted in a great increase of satellite cell proliferation [8–11]. More recently, it has also been reported that blood-borne macrophages play an essential role in the grafting and proliferation of muscle transplants in vivo [12]. Our experiments have suggested that the factor released by macrophages was a water-soluble molecule that displayed the characteristic chemical properties of cytokines [10]. This factor has up to now been only partially purified, mainly due to its low abundance in a complex mixture, the cell culture medium, that contains high levels of serum proteins [13].

In this paper we now show that a murine macrophage cell line (J774) can release a muscle-specific growth factor when cultured in serum-free medium. Supplementing cultures of rat and human primary myoblasts with such macrophage-conditioned medium (MCM) led to a marked increase in the percentage of MyoD-positive cells and, eventually, to an increased number of myotubes. Experiments have also been performed to test the effect of MCM administration in vivo; preliminary results indicated that MCM significantly enhance the regeneration processes in muscles damaged by tissue ablation. The possibility of obtaining large amounts of MCM in the total absence of serum now opens the way for a

much easier biochemical characterization of the muscle-specific factor released by the J774 cells. This in turn will pave the way to its use in the development of therapeutic protocols.

Materials and methods

Cell culture procedures

The J774 murine macrophage cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (growth medium). All cell culture reagents were from Sigma-Aldrich. To prepare the macrophage-conditioned medium (MCM), confluent cells were washed three times in phosphate-buffered saline (PBS) and re-fed with serum-free medium containing 0.01 mg/ml lipopolysaccharide in order to activate the macrophages. After 36 hours MCM was collected, centrifuged at 400xg and stored at -20°C.

Primary rat myoblasts were obtained from newborn (1- to 2-day-old) Wistar rats using the trypsinization procedure described elsewhere [10]. After a one-hour pre-plating, that eliminated most non-muscle cells, cell suspension was cultured in plastic 24-well plates at a density of $12x10^4$ cells per well in growth medium. Wells contained a glass coverslip coated with gelatin. Where indicated, 12 hours after seeding, the growth medium was supplemented with MCM (20% final, v/v). Myotube formation was induced by replacing the growth medium with fusion medium, containing horse serum (HS, 2% final) instead of FBS.

Fibroblasts and fibroblast-like cells were obtained from the pre-plating step carried out during the myoblast enrichment and cultured in growth medium. In order to eliminate the myotubes that had formed from the few contaminating myoblasts, cells were trypsinized 7 days after seeding and re-plated in 24-well plates on gelatin-coated glass coverslips.

Primary human myoblasts were a generous gift of V. Mouly (INSERM, Paris, France). Cells were obtained from the quadriceps muscle of a 35-week-old fetus and were cultured in F12 medium (Life Technologies) supplemented with 10% FBS and gentamicin (50 μg/ml).

Cell staining procedures

For desmin/propidium iodide staining, cultures were washed twice with PBS, fixed with cold acetone for 10 min, air-dried and then incubated for 60 min at 37°C with an anti-desmin monoclonal antibody (mAb) (Roche Molecular Biochemicals) diluted 1 to 5 in phosphate buffer containing 0.5% bovine serum albumin. A FITC-labeled anti-mouse IgG secondary antibody (Roche Diagnostic) was used to reveal positive muscle cells. Nuclei were counterstained with propidium iodide (1 mg/ml in PBS, containing 100 μg/ml DNAse-free RNAse A) for 30 min at 37°C. Coverslips were then mounted and observed with a Zeiss Axiovert epifluorescence microscope.

For MyoD/Hoechst 33258 staining, at the indicated times cultures were washed twice with PBS, fixed with paraformaldehyde (2% in PBS) for 20 min and treated with 50 mM NH_4Cl for 20 min (to quench autofluorescence). Cells were then permeabilized with Triton (0.5% in PBS) for 10–15 min and incubated overnight at 4°C with rabbit anti-MyoD polyclonal antibody (Santa Cruz Biotechnology). After PBS washing, a rhodamine-conjugated goat anti-rabbit secondary antibody was used (DAKO). Finally, nuclei were labeled with Hoechst dye 33258 (0.1 μg/ml in PBS, Sigma-Aldrich) for 5 min at room temperature and coverslip-mounted.

For myogenin/Hoechst 33258 staining, we followed a procedure identical to that for MyoD/Hoechst 33258 staining, except that we used a rabbit anti-myogenin polyclonal antibody (Santa Cruz Biotechnology).

Cell proliferation assay

At the desired time points, bromo-deoxyuridine (BrdU, Roche Molecular Biochemicals) was added to the culture medium to a final concentration of 100 μM. After one hour cells were rinsed in PBS and fixed in paraformaldehyde (2% w/v in PBS) at room temperature for 15 min. After NH_4Cl treatment, cell membranes were permeabilized with Triton X-100 (0.5% v/v in PBS) and nuclear DNA was fragmented by acidification in 1M HCl. Cells were then stained with a monoclonal antibody against BrdU (Roche Diagnostic), at the dilution of 1:5, followed by a secondary FITC-conjugated goat anti-mouse (Roche Molecular Biochemicals). Nuclei were counter-stained with 4',6'-diamino-2-phenylindole dihydrochloride (DAPI) (0.1 μg/μl in PBS).

Evaluation of immunohistochemistry experiments

The percentages of BrdU-, MyoD-, myogenin-, and desmine-positive cells on the total nuclei were evaluated at the indicated times by counting 6 different microscopic fields per time point. Values were averaged between four different experiments.

Induction of muscle damage and MCM administration

Tibialis anterior (TA) muscles from eight 200-g, male Wistar rats were exposed and approximately 80% of the tissue was removed, paying attention to leave the tendinous ends intact. The cavity left by the operation was filled with inert surgical material (fragments of fibrin sheets) and the external muscle fasciae and the skin were sutured separately. Twenty-four and forty-eight hours after surgery, half of the treated limbs were injected with 500 μl MCM. Animals were sacrificed by cervical dislocation at the indicated times and the TA muscles were harvested and snap-frozen in liquid nitrogen. Serial sections obtained with a cryostat were H&E stained and immunostained with an anti-fetal myosin antibody (a generous gift of Prof. S. Schiaffino, Padua). Experiments were carried out following the specifications of the law "D.L. 27-1-92, number 116, *Circolare Applicativa del Ministero della Sanità*, numero 8 del 22-4-1994".

Results

Effect of MCM on myoblast proliferation and differentiation

The first set of experiments was performed on rat primary myoblasts. Cultures were followed for up to 5 days, from the time the myoblasts were seeded until they had fused into

myotubes. Cells were kept in MCM-enriched medium for 60 hours, starting 12 hours after plating, and then switched to fusion medium. The presence of MCM did not have any effect on the proliferation rate of MyoD-negative cells, whereas it induced a 77% increase in the percentage of MyoD-positive proliferating myoblasts (from 13% to 23%, Fig. 1).

The presence of MCM was associated with a diminished expression of myogenin for the first 72 hours (Fig. 2). On the other hand, after switching to the fusion medium, MCM-treated cells showed a marked increase in the number of myogenin-positive nuclei at both 108 and 120 hours (Fig. 2).

In order to determine if the factors secreted by J774 cells were actually muscle-specific, we analyzed the effect of MCM on primary fibroblasts and fibroblast-like cells. Again, BrdU was added to the culture medium 48 hours after plating (i.e. 36 hours after adding MCM). This time, no significant difference was found between MCM-treated and control cells (42%±2% vs. 40%±2% of BrdU-positive cells, respectively).

Effect of MCM on human primary myoblasts

Despite their fetal origin, human myoblasts have a lower proliferation rate and a longer differentiation time than their rat counterpart; for this reason the experiments were carried out for a longer time span. Cells were analyzed by desmine staining 3, 5, 7 and 10 days after seeding; again, the presence of MCM in the culture medium increased by circa 40% the percentage of myogenic (i.e, desmine-positive) cells compared to untreated controls (Table 1).

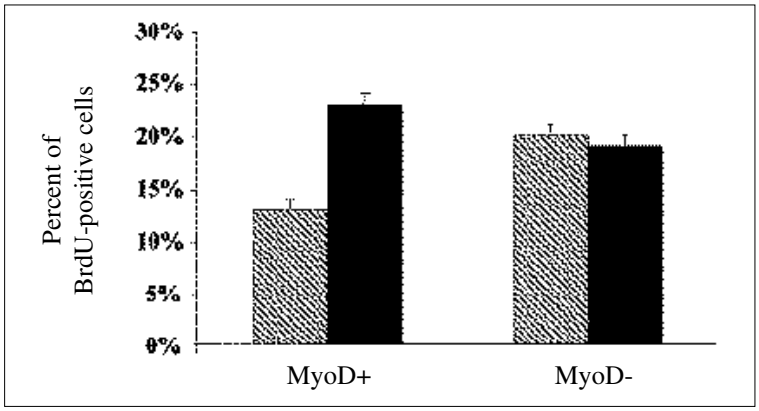

Fig. 1 Effect of macrophage-conditioned medium (MCM) on myoblast proliferation. The chart shows the percentages of MyoD-positive (*MyoD+*) and MyoD-negative (*MyoD-*) nuclei in relation to BrdU-positive cells. *Black bars*, MCM-treated cells; *shaded bars*, untreated cells

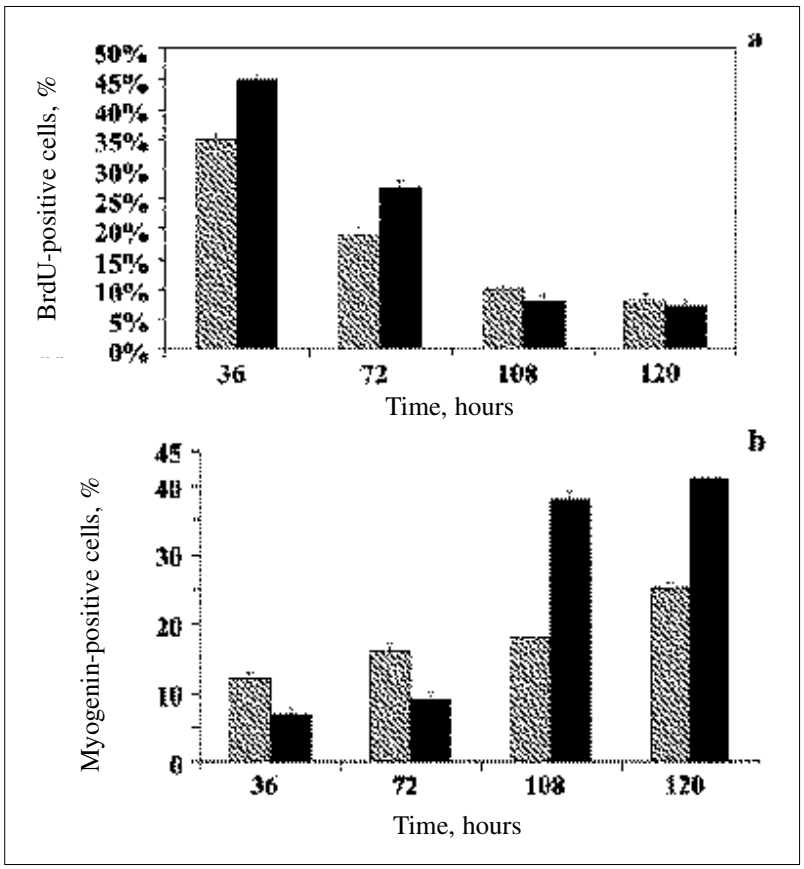

Figure 2a, b Effect of macrophage-conditioned medium (MCM) on myoblast differentiation. Cells were kept in either growth medium or MCM-enriched medium for the first 72 hours and then switched to fusion medium. Cells were fixed at the indicated times after seeding. **a** BrdU-positive cells. **b** Myogenin-positive cells. *Black bars*, MCM-treated cells; *shaded bars*, untreated cells

Table 1 Effect of macrophage-conditioned medium (MCM) on human primary myoblasts proliferation. Values are mean (SD)

Days after plating	Desmin-positive cells, %	
	Control	MCM-treated
3	40 (2)	48 (4)
5	42 (3)	57 (2)
7	48 (2)	67 (2)
10	51 (6)	72 (4)

Effect of MCM on muscle regeneration

MCM activity was also tested in vivo, in a model of extensive muscle regeneration induced by tissue ablation. Approximately 80% of the muscle mass was surgically removed from the tibialis anterior muscles of adult rats and 500 µl MCM was injected 24 and 48 hours after surgery in half of the limbs, leaving the others as controls. Immunohistochemical analysis showed that seven days after surgery the muscles that received MCM showed a substantial increase in the number and size of regenerating fibers compared to non-injected controls (Fig. 3A, B). At 14 days, H&E staining clearly showed that the treated muscles had virtually completed the regeneration process (most of the fibers were of normal size and only a few mononucleated cells were still present), whereas in the untreated samples the regeneration was still underway (Fig. 3C, D). Also, the treated muscles had recovered a much higher amount of muscular mass compared to untreated controls, 159±4 mg vs. 88±3 mg, respectively. The muscle weight of the non-operated, age-matched controls was on average 250 mg.

Discussion

We previously reported that the co-culture of macrophages from rat peritoneal exudate with embryonic myoblasts led to a marked increase in the number of myotubes in the differentiated cultures [8, 10]. In this paper we now report that the macrophage cell line J774 can also secrete a factor that exhibits the same properties as that secreted by peritoneal macrophages. The effect of J774 MCM was tested not only on rat primary myoblasts but also, for the first time, on human primary myoblasts. We also report that this factor is active not only in vitro but also in vivo.

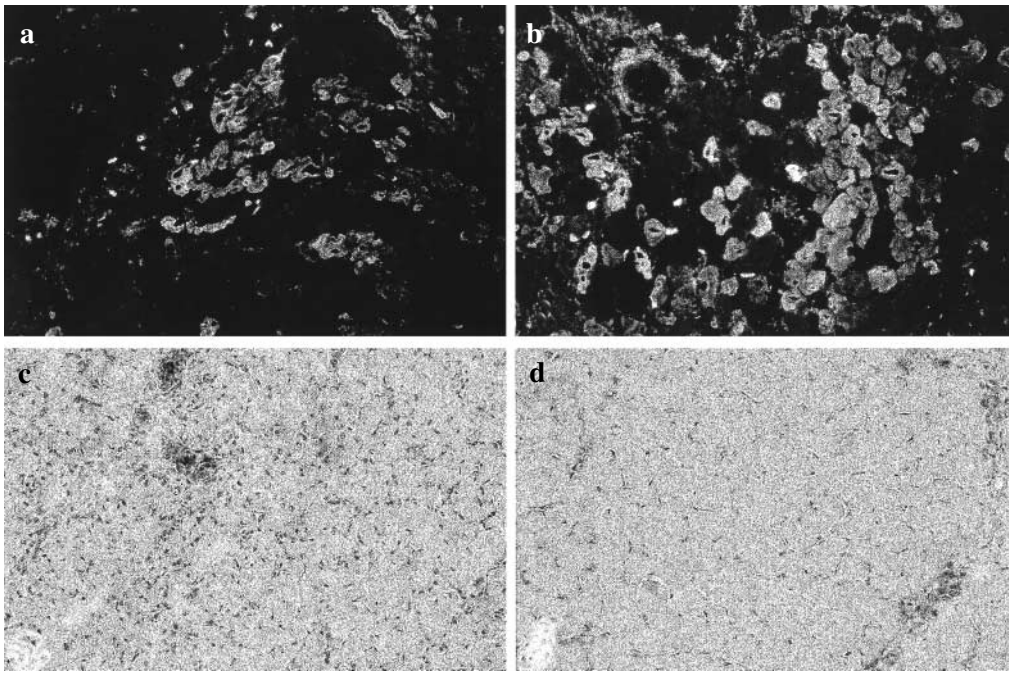

Figure 3a-d Effect of macrophage-conditioned medium (MCM) on muscle reconstruction in vivo. **a**, **b** Sections prepared seven days after muscle damage, stained with an anti-fetal myosin antibody (**a**, control; **b** MCM-treated). **c**, **d** Sections prepared 14 days after muscle damage, H&E staining (**c**, control; **d**, MCM-treated)

Our first step was to design a protocol to grow the cells in the absence of serum. Our data indicated that primary macrophages could be kept in serum-free conditions for up to 48 hr [14]. More recent experiments showed how J774 cells could also be maintained in serum-free medium for 36 hr without inducing any significant toxicity (unpublished results). Replacing peritoneal macrophages with a cell line expanded in serum-free conditions provided two major advantages: a greater supply of macrophage-conditioned medium without the need to use animals and, even more relevant, the absence of serum facilitated the future biochemical characterization of the MCM components. In our first set of experiments we observed the effect of J774 MCM treatment on the proliferation and differentiation of primary rat myoblasts, following the expression of two muscle-specific transcription factors (MyoD and myogenin). The former is expressed in determined myoblasts right after the induction to proliferate and its presence declines when the cells start to differentiate into myotubes. The latter has the opposite behavior, in that it is expressed only after the beginning of differentiation [15].

Our results showed that when primary cultures were treated with MCM, the amount of proliferating determined myoblasts (i.e. positive for BrdU incorporation and MyoD staining) increased by 77% compared to the controls. This value is comparable to those found in our previous experiments with peritoneal macrophages [10]. Importantly, the effect of J774 MCM was limited to determined muscle cells, i.e. the percentage of proliferating MyoD-negative cells was not affected by the presence of macrophage-conditioned medium. This finding was also confirmed in cultures of partially purified primary fibroblasts. It is worth mentioning that in primary muscle cultures there are also myogenic cells that do not express MyoD [15] and therefore one can not rule out the possibility that the factor secreted by J774 cells might lead to an increased number of MyoD-positive cells by favoring the transition from MyoD- to MyoD+.

MCM also appeared to slow down the differentiation process; in fact, during the first 72 hours from plating we found a decreased number of myogenin-positive nuclei in MCM-treated cultures compared to controls, indicating that fewer muscle cells had entered the differentiative path. Once the myoblasts were induced to differentiate by switching to fusion medium (that did not contain MCM), the higher number of myoblasts present in MCM-treated cultures translated into a higher number of myogenin-positive cells.

When tested on human primary myoblasts, MCM treatment led to an approximately 40% increase in the percentage of desmin-positive cells. Such effect, albeit not as high as that found in rat myoblasts, clearly indicated that the myogenic factor produced by J774 cells is not species-specific.

Finally, our initial in vivo data indicate that MCM also exerts a significant effect on muscle regeneration processes following tissue ablation. In fact, the presence of MCM during the first 48 hours after muscle damage led to a higher percentage of newly formed myofibers at 7 days and to a significant increase in the recovery of the total muscle mass after two weeks (from 35% to 64%). Experiments are presently in course to determine the exact nature of MCM action. In view of our in vitro findings, we hypothesize that an increased proliferation of activated satellite cells could be responsible for the improved muscle repair.

Gene therapy and tissue engineering are both becoming increasingly attractive perspectives in the field of muscle disorders [16]. The findings presented here indicate that J774 MCM is a convenient source of myogenic factors. Once purified, this factor will be an important tool for the design of therapeutic protocols in which an increased proliferative capacity of myoblasts is desirable, such as myoblast transplantation, ex vivo gene transfer and muscle repair.

Sommario *Obiettivo del presente lavoro è stato determinare se la linea di macrofagi murini J774 possa essere utilizzata per la produzione di fattori di crescita muscolo-specifici. A tale fine, cellule della linea di macrofagi murini J774 sono state cresciute in assenza di siero ed il terreno di coltura (MCM, macrophage-conditioned medium) è stato poi utilizzato per trattare colture di mioblasti primari in vitro e muscolo rigenerante in vivo. Gli effetti dell'MCM in vitro sono stati verificati analizzando l'espressione di proteine muscolo-specifiche, in parallelo ai processi di proliferazione e differenziazione cellulare. I fattori contenuti nell'MCM hanno notevolmente aumentato il potenziale proliferativo di mioblasti primari di ratto ed umani ed il loro effetto è risultato altamente muscolo-specifico. Somministrato in vivo, l'MCM ha migliorato l'efficienza dei processi rigenerativi in muscolo scheletrico danneggiato per ablazione. La possibilità di ottenere grandi quantità di MCM privo di siero è un passo fondamentale verso la caratterizzazione biochimica dei fattori miogenici in esso contenuti ed il loro successivo utilizzo in protocolli di terapia genica ex-vivo e di riparazione muscolare.*

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