

Myo-D Positive Cells Overcome Fibroblasts in Primary Muscle Cultures Grown in the Presence of a 50-10 kDa Cytokine Secreted by Macrophages

Emanuele Giurisato, Luciano Dalla Libera⁽¹⁾, Barbara Ravara⁽¹⁾, Maria L. Massimino and Marcello Cantini

CRIBI Biotechnology Center and Department of Experimental Biomedical Sciences and (1) CNR Unit for Muscle Biology and Physiopathology c/o Department of Experimental Biomedical Sciences, University of Padova, Padova, Italy

Abstract

Recently we demonstrated that in an *in vitro* culture where both myoblasts and macrophages were grown together, the myotube formation is strongly increased by the presence of an acid stable, heat-labile, soluble growth factor(s) secreted by macrophages. We now present new data concerning the culture medium and the role of macrophages in myoblast proliferation, and in particular we demonstrate that: i) macrophages also release the factor in a totally serum-free medium; ii) muscle growth induced by macrophages is mainly the consequence of an increased myoblast proliferation as revealed by the presence of an increased number of MyoD-positive myonuclei, while fibroblasts show no increase; iii) the muscle specific cytokine(s) released by activated macrophages has a molecular mass in the range 50-10 kDa as determined by both microfiltration and chromatographic experiments.

Key words: macrophage cytokines; MyoD; muscle regeneration; heparin affinity chromatography; myoblast cultures.

Basic Appl. Myol. 8 (5): 381-388, 1998

In response to a variety of injuries such as those caused by mechanical damage or by chemical or biological agents adult skeletal muscle is able to regenerate by the activation of a special type of cells, named satellite cells, which in only a few days after injury strongly proliferate and fuse to form new myofibres [8, 9, 33]. The success of correct muscle repair is related to the size of the injury, since an extensive destruction of the external lamina of muscle fibres favours the formation of connective tissue. The successive innervation of the newly formed fibres results in a new functional muscle [1, 23, 36, 46]. Muscle destruction and successive regeneration is accompanied *in vivo*, during the early stages, by an inflammatory process in which macrophages play a key role in muscle integrity reconstitution, at first by removing the cellular debris [25, 41, 43]. As macrophages are known to produce a variety of cytokines responsible for mitosis activation and chemotaxis of a variety of cells ([44], for a review see ref. [26, 37]), in recent years we have grown myoblasts (from both man and rat) in the presence of macrophages obtained from a peritoneal

exudate: the result was a great increase of satellite cell proliferation. Successive experiments have suggested that the growth factor(s) released by macrophages is a soluble factor that displays the characteristic chemical properties of cytokines [11, 12]. This factor has up to now been only partially purified mainly due to its low abundance in a complex mixture, such as cell culture medium that contains very high levels of serum proteins (for instance, from fetal calf serum) [17].

Recent studies in a rat model showed that in the injured muscle macrophages infiltrating necrotic fibres are a population distinct from those accumulated during the regeneration of muscle fibres [18, 34, 49]. In addition we demonstrated that this population, that is immunologically characterized as an ED2+ macrophage subpopulation, is really involved in the increased proliferation of myoblasts [32].

With regard to the myogenic specific growth factors released directly by macrophages or induced by them from other cells present in the damaged site, such as endothelial cells and/or fibroblasts, of particular interest are known growth factors such as fibroblast growth

MyoD+ cells increase in presence of macrophage cytokines

factor (FGF) [2, 16, 24, 38, 42, 47], insulin-like growth factor (IGF) [20, 21], transforming growth factor- β (TGF β) [31, 40], interleukine-6 (IL6) [5, 7, 13], leukemia inhibitory factor (LIF) [4, 6], platelet-derived growth factor (PDGF) [52, 53], colony stimulating factor-1 (CSF-1) [27], hepatocyte growth factor (HGF) [3] which have been demonstrated to have specific effects on muscle growth *in vitro* and more recently also *in vivo* (for a review see [10, 20, 26, 37, 39]).

In this paper we present new data concerning the role of macrophages in myoblast proliferation, in particular we demonstrate: i) macrophages also release the factor in a totally serum-free medium; ii) the effective increase of MyoD positive cells in primary muscle cultures, with respect to fibroblasts which show no increase; iii) the muscle specific cytokine released by activated macrophages has a molecular mass in the range 50-10 kDa as determined by microfiltration and chromatographic experiments.

Materials and Methods

Macrophage cultures

Macrophages were obtained from rat peritoneum after the injection of thioglycollate broth. The fluid was collected one day later, centrifuged at 400xg and the cell sediment was resuspended in a complete medium (Dulbecco's modified Eagle medium (DMEM)) added with 10% fetal calf serum (FCS). Cells were seeded in flasks and after adhesion, cultures were washed three times by shaking with phosphate buffer solution (PBS) to remove non-adherent cells. Adherent cells (mainly macrophages) were cultured in i) serum-containing (10% FCS) DMEM (scMCM) or ii) serum-free DMEM (sfMCM). The culture medium (macrophage conditioned medium, MCM) was harvested at 48 hr [11].

Membrane fractionation of MCM

To identify the range of molecular mass of the growth factor present in MCM we sequentially fractionated the medium by a membrane filtration system (Sartocon micro) in four fractions characterized by the following nominal molecular mass: >100, 100-50, 50-10, <10 kDa. Each fraction obtained was restored to the initial volume and used in a myoblast proliferation assay as described below.

Primary muscle cell cultures

Myoblasts were obtained from the legs of euthanized newborn rats (Wistar) by enzymatic digestion with trypsin. Cell suspension, after cell pre-plating to eliminate most fibroblasts, was cultured in plastic 12-well plates at a density of 10^5 cells per cm^2 in DMEM supplied with 10% FCS, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin for 60h (before myoblast fusion) and for 5 days (after myoblast fusion to form myotubes).

The wells contain a glass coverslip coated with gelatin. This culture was used as control. Identical sets of primary muscle cell cultures were grown in complete medium supplied with 20% of MCM or fractions obtained after filtration on membrane system or fractions derived from chromatographic experiments (we added MCM only once, at the beginning of the experiment).

Muscle cell staining

Desmin/propidium iodide

Five-day-old differentiated muscle cell cultures (myotubes) were washed twice with PBS and fixed with cold acetone for 10 min, air-dried and then incubated for 60 min. at 37 °C with an anti-desmin mAb (Boehringer, U.S.A.) diluted five times with 0.5% BSA in PBS. An anti-mouse IgG secondary antibody fluorescein-labeled was used to reveal differentiated myotubes.

To highlight nuclei we stained the cultures with propidium iodide (PI) 4 $\mu\text{g}/\text{ml}$ in PBS containing 100 $\mu\text{g}/\text{ml}$ of DNase-free RNase A for 30 min at 37 °C according to [32]. Cultures were observed under a fluorescence microscope: both myotubes and PI positive nuclei into myotubes were counted.

MyoD/Hoechst No 33258

Primary muscle cell cultures before myoblast fusion (60 hours) were washed twice with PBS, and fixed with paraformaldehyde 2% in PBS for 20 min. After PBS washing and quenching with NH_4Cl 50 mM for 10 min, cells were permeabilized with Triton 0.5% in PBS for 10 min. Cells were incubated overnight at 4°C with a rabbit anti-MyoD polyclonal antibody specific for skeletal muscle nuclei (Santa Cruz Biotechnology, USA). After PBS washing a rhodamine-conjugated secondary antibody was used. Cultures were observed by fluorescence microscope to reveal MyoD positive nuclei.

After Myo D staining cells were labeled with Hoechst No 33258 diluted in PBS for 5 min at room temperature, washed twice with PBS and mounted in Elvanol. This second staining allows us to count the number of total nuclei in cultures. Slides were observed by fluorescence microscopy.

Myoblast proliferation assay

Control cultures, scMCM, sfMCM treated muscle cultures and membrane-fractions derived treated cultures before (60 h) myoblast fusion were used. In unfused muscle cultures both MyoD positive nuclei and positive Hoechst nuclei were counted. Three different experiments were performed and 6 different microscopic fields were analyzed for each kind of experiment. Statistical significance was calculated using the Student's t-test.

MyoD+ cells increase in presence of macrophage cytokines

Heparin-sepharose affinity chromatography

MCMs (25 ml) were applied to a HiTrap Heparin column (5 ml) (Pharmacia, Uppsala, Sweden), equilibrated with buffer PBS, flow rate 2 ml/min. After the sample was loaded the column was washed with buffer PBS until the UV absorbance at 280 nm returned to baseline (unretained material). The factor was eluted with a step of PBS containing 0.5 M NaCl and about 3 ml were obtained (retained material). The column was washed with high salt (2M NaCl) before re-use; in this step no protein was released and detected.

High-performance liquid chromatography

250 ml of MCMs, unretained and retained material by Heparin column were loaded on a Superdex 75 HR 10/30 column (300 x 10 mm I.D.) (Pharmacia, Uppsala, Sweden) connected to a Bio-Rad (Richmond, CA, USA) high-performance liquid chromatographic system, equipped with a Model UV-1806 variable-wavelength detector from Bio-Rad (set at 220 nm) and a Rheodyne Model 7125 sample injector (1-ml loop). The eluent was PBS at a flow rate of 1.0 ml/min.

The water used for chromatography was HPLC-grade and all solutions were degassed and passed through a 0.45- μ m Millipore filter prior to chromatography. All reagents used were of analytical-reagent grade.

SDS PAGE

The one dimensional SDS PAGE of MCM and of the fractions from it derived was carried out according to [29]. Electrophoresis was carried out in 12.5 % polyacrylamide gel. Slabs were stained with the sensitive silver method [35].

Results

We have previously demonstrated that rat peritoneal exudate contains a variety of cells, mainly macrophages that are involved in the secretion of muscle growth factors when co-cultured with myoblasts. In addition we also observed an increased muscle growth in muscle cultures treated with MCM [11, 12, 32]. In our opinion the increased muscle growth in culture is a consequence of an increased myoblast proliferation. Indeed the number of PI positive nuclei into myotubes grown in the presence of MCM, both serum-containing and serum-free medium, is increased (Fig. 1). To eliminate the possibility that MCMs favour the fusion of myoblasts instead of stimulate their proliferation we counted the MyoD positive nuclei (myoblasts) before fusion (60 hours). Figure 2 shows a typical experiment. The number of MyoD+ nuclei (myoblasts) markedly increased in both MCMs treated cultures as compared to controls. It is evident that MyoD+ nuclei almost doubled in treated cultures. In table Ia are summarized the results of three different experiments.

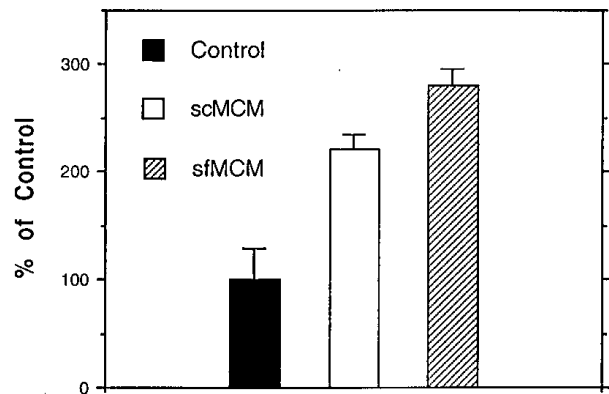


Figure 1. Percentage of PI positive myonuclei present in 5-day myotube cultures in presence of control medium; scMCM, conditioned medium serum-containing; sfMCM, conditioned medium serum-free. The cultures were labeled with PI and anti-desmin antibody.

Total nuclei were also counted: by subtracting MyoD+ nuclei number (panels a, c, e) from total nuclei number (panels b, d, f) we obtained the number of fibroblast-like cell nuclei (Table Ib). This value is almost similar in treated and control cultures.

Since both scMCM and sfMCM gave the same results in terms of myoblast increased proliferation we used sfMCM to avoid the high levels of interfering serum proteins in the experiments described below.

For the identification of the factor involved in increased muscle cell proliferation, in the first step we tried to separate by means of a filtration system sfMCM in distinct fractions containing proteins with Mw: >100, 100-50, 50-10, <10.

Biological assays on muscle cultures with molecular weight defined fractions were performed by counting PI positive nuclei into desmin labeled myotubes (a technique faster and more easy than MyoD staining). Results are shown in Fig. 3. It is evident that the myo-mitogenic factor(s) is present in the fraction characterized by Mw in the range 50-10 kDa. The number of fibroblasts did not increase.

We have already demonstrated that the factor involved in increased muscle cell proliferation has a strong affinity for heparin like many growth factors [17]. The fractions of sfMCM obtained after affinity chromatography on HiTrap Heparin column, both retained and unretained ones, were analyzed in detail by means of size-exclusion chromatography. Superdex 75 HR, a crosslinked agarose-based medium is particularly suitable for high-performance gel permeation of biomolecules, characterized by a molecular mass operating range from 1 to 70 kDa. Calibration results were as follows: bovine serum albumin with Mr 67 kDa, 7.5 min; lactalbumin with Mr 35 kDa, 8.5 min; cytochrome c with Mr 12 kDa, 12 min; vitamin B12

MyoD+ cells increase in presence of macrophage cytokines

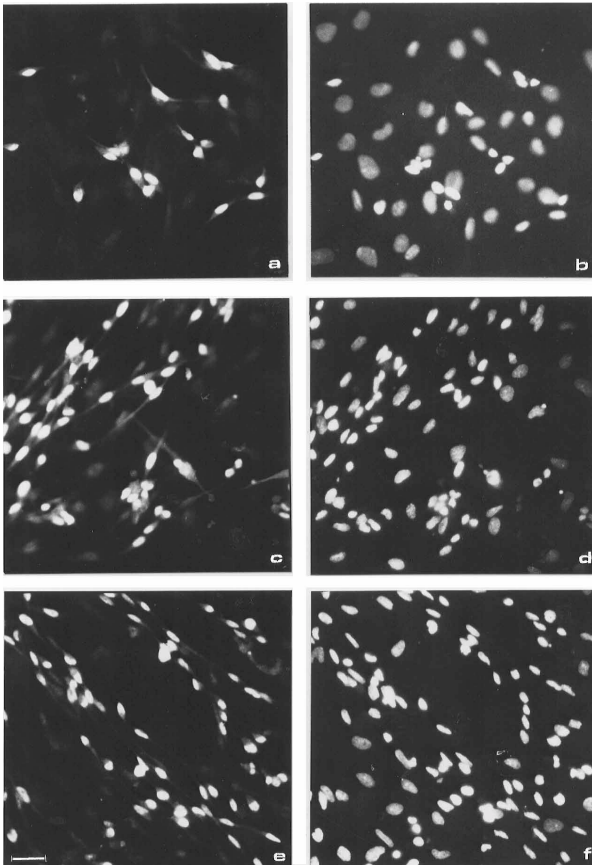


Figure 2. scMCM and sfMCM treated primary muscle culture after 60 hours. Cultures were stained with anti MyoD polyclonal antibodies (a, c, e) and sequentially with Hoechst No 33258 (b, d, f). Control (a, b); scMCM treated cultures (c, d); sfMCM treated cultures (e, f). Bar = 29 μ m.

with Mr 376 Da, 23 min. Figure 4 shows the three chromatograms obtained from sfMCM, heparin-retained and heparin-unretained material. The chromatograms of sfMCM and heparin-unretained material are characterized by the presence of several peaks and are indeed very similar (Fig. 4, panels a and b). By contrast the chromatogram of heparin-retained material (Fig. 4 panel c) contains less peaks the majority of which have retention times corresponding to a molecular mass range 50-10 kDa. When examined by SDS-PAGE these column fractions (peaks A, B, C and D) revealed the presence of proteins characterized by a molecular mass range 50 to 10 kDa (Fig. 5). Peaks E and F did not contain sufficient material to be revealed even by the highly sensitive silver staining and therefore are not shown.

Discussion

During muscle regeneration macrophages infiltrate the muscle at the site of damage to phagocytose necrotic tissue and secrete growth factors which stimulate the

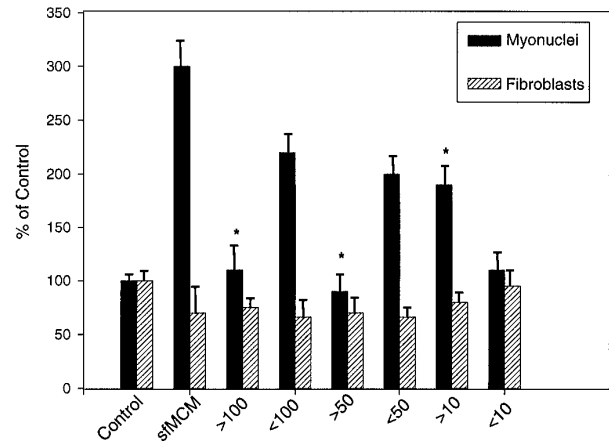


Figure 3. Percentage of nuclei desmin-positive and fibroblast-like nuclei present in myotubes at 5 days in presence of sfMCM fractions obtained by microfiltration. In fractions 50 to 10 kDa (asterisks) it is evident myoblast enhanced proliferation.

satellite cell proliferation [22, 25, 36, 41]. In rat it has been demonstrated that an ED2+ macrophage subpopulation is responsible for this event [25, 32]. So far as the relationship between satellite cells and macrophages during muscle regeneration is concerned, we showed that cultures obtained from injured muscle contain almost exclusively proliferating myoblasts and that myoblast proliferation *in vitro* is maximally activated when macrophage infiltration *in vivo* reaches its peak [12, 14]. We have also shown that macrophages co-cultured with either embryonic myoblasts or satellite cells increase myotube formation *in vitro* by secretion of growth factor(s) [11, 12].

In this study we present evidence that growth factor(s) released by macrophages is characterized by a molecular mass range 50 to 10 kDa and is specifically involved in mitosis regulation of myoblasts increasing MyoD positive cells *in vitro* as compared to fibroblast-like cells, which do not increase.

The increased muscle growth induced by macrophages may be a consequence of either an accelerated myoblast fusion (differentiative effect) or an increased myoblast proliferation (proliferative effect) or both. To answer this question in the past we have estimated the number of myoblasts by counting MyoD positive nuclei [32, 50, 51] in muscle cell cultures grown in the presence of MCM before myoblast fusion. Here we confirm the highest myonuclei (MyoD+) number in treated muscle cultures before myoblast fusion and, in addition, we show that the number of fibroblast-like cells does not vary significantly in MCM treated cultures. These results support our hypothesis that activated macrophages possess a muscle specific proliferative effect. Kaufman's laboratory showed that MyoD negative myoblasts could be observed in primary rat

MyoD+ cells increase in presence of macrophage cytokines

Table I. Percentage of MyoD+ positive nuclei and fibroblast-like nuclei present in 60 h primary muscle cell cultures (before fusion).

(a) MyoD+ nuclei					
	Control	MCM(sc)	MCM(sf)	Δ MCM(sc)%	Δ MCM(sf)%
Exp.1	18 $\pm$ 4	46 $\pm$ 8	61 $\pm$ 8	155	238
Exp.2	23 $\pm$ 7	52 $\pm$ 7	63 $\pm$ 10	126	173
Exp.3	20 $\pm$ 6	50 $\pm$ 9	59 $\pm$ 8	150	195
				Δ =143	Δ =202

Note. Results are the mean of six different microscopic fields for each experiment. It is evident that myonuclei strongly increase in both MCM(sc) and MCM(sf) treated cultures as compared with controls.

(b) Fibroblast-like cell nuclei					
	Control	MCM(sc)	MCM(sf)	Δ MCM(sc)%	Δ MCM(sf)%
Exp.1	28 $\pm$ 7	33 $\pm$ 9	35 $\pm$ 7	18	25
Exp.2	31 $\pm$ 8	37 $\pm$ 7	39 $\pm$ 8	19	26
Exp.3	28 $\pm$ 6	35 $\pm$ 6	37 $\pm$ 7	25	32
				Δ =21	Δ =28

Note. Results are the mean of six different microscopic fields for each experiment. It is evident that fibroblast-like cell nuclei are almost similar in both MCMs and in control cultures.

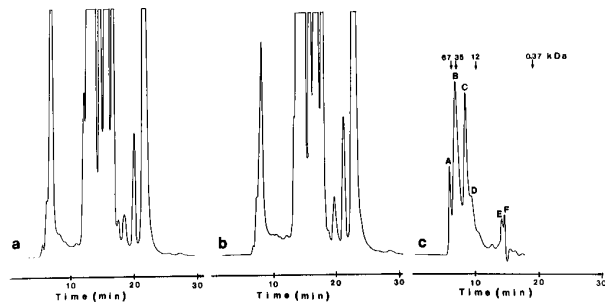


Figure 4. Chromatograms obtained on a Superdex 75 column with detection at 220 nm. Eluent: PBS; flow-rate: 1.0 ml/min. (a) 150 mg of sfMCM; (b) 120 mg of fraction unretained by heparin column; (c) 30 mg of fraction retained by heparin. In panel (c) letters label the major peaks; arrows indicate the elution position of molecular mass markers. Absorbance full scale is 0.1 in (a) and (b), 0.005 in (c).

cultures [28]. At the light of this observation the MyoD positive cells we counted did not represent all myoblasts and therefore in our experiments myoblasts would be underestimated. We are aware that the MCM could favour the transition MyoD-/MyoD+ (differentiative effect) with a final increased number of MyoD positive cells. However we reported that clonal assays using MCM treated satellite cells demonstrated a proliferative effect [12]. Taken together, all the reported data support our hypothesis on proliferative effect of MCM instead of

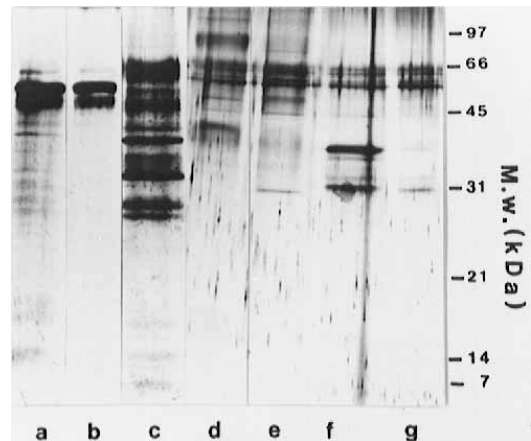


Figure 5. SDS-PAGE of Superdex 75 derived fractions. (a) sfMCM; (b) heparin-unretained material; (c) heparin-retained material; (d) peak A; (e) peak B; (f) peak C; (g) shoulder D. (a), (b), (c): 8 mg; (d) to (g): 0.2 mg. Gel was stained by the silver technique.

differentiative effect. To definitively clarify this point additional experiments are necessary.

Since our final goal is to isolate and characterize this myo-mitogenic factor, we have cultured exudate macrophages in a serum-free medium to eliminate interfering serum components. In fact our standard macrophage-conditioned culture media contained many

MyoD+ cells increase in presence of macrophage cytokines

peptide components, some of which are serum components, while others are secreted by cells themselves. Therefore we repeated the experiments using a MCM obtained from macrophages cultured in serum-free medium. In these latter conditions the increased number of MyoD positive cells was still observed and was in fact even greater than with MCM derived from macrophages cultured in the presence of serum. It has been reported that the majority of primary cultures grown *in vitro* need serum components (growth factors) for survival and proliferation and it has also been reported that myoblasts cultured in a serum-free medium undergo apoptosis [19, 30, 45, 48]. Our experiments, however, demonstrate that macrophages cultured in a serum-free medium, not only survive for some days but also produce a MCM highly mitogenic for myoblasts.

In order to obtain information about the molecular mass of the muscle specific factor we sequentially fractionated the sfMCM by a membrane filtration system. The myo-mitogenic factor is present in the fraction characterized by a Mw in the range 50 to 10 kDa, which is typical for known growth factors. Our data also demonstrate that the MCM active fraction is able to induce myoblast and not fibroblast overstimulation in the same culture.

The electrophoretic analyses of fractions obtained by the microfiltration system failed to give clear results about the precise molecular mass of the proteins they contained. In fact molecular mass cutoff of the membranes used is only nominal. Due to this drawback some parameters such as protein concentration, protein-protein interaction, size or conformation of the molecule may affect the membrane performance. Therefore in parallel we analyzed the MCM by means of size-exclusion chromatography.

Since we and other have demonstrated that heparin has strong affinity for myoblast mitogens [15, 17] we here show that in the fraction retained by the heparin column, proteins of molecular mass range 50 to 10 kDa are indeed present, as revealed by both electrophoretic and size-exclusion chromatography experiments. The abundance of this factor can be roughly determined as 0.04 - 0.08 mg/ml, from the amount of medium utilized for the purification and from the estimated sensitivity of the silver staining method used for detection of proteins on SDS-PAGE gels (0.05 - 0.1 mg/band).

We do not know whether this mitogen released by macrophages is one of the known growth factors or an as yet unidentified one. Further experiments are needed in order to clarify this point.

In conclusion these results stimulate further researches to characterize more precisely the nature of this muscle growth factor with a view to using it to selectively increase myoblast proliferation in situations such as

muscle repair, muscle cell transplantation and gene therapy via myoblasts.

Acknowledgements

This study was supported in part by funds from the Italian MURST 60% to M.C. and from Italian C.N.R. to the Unit for Muscle Biology and Physiopathology. E.G. is the recipient of a fellowship from Banca Popolare Vicentina and Associazione Industriali di Vicenza. The financial support of Telethon-Italy to the project "Myoblast-selective mitogen(s) released by macrophages as a tool for muscle regeneration and gene therapy" (Grant n. A.101 to L.D.L.) is gratefully acknowledged.

Address correspondence to:

Dr. Marcello Cantini, Department of Experimental Biomedical Sciences, University of Padova, Via G. Colombo 3, I-35121 Padova, Italy, phone +39 49 8276068, fax +39 49 8276049, Email cantini@civ.bio.unipd.it.

References

- [1] Allbrook D: Skeletal muscle regeneration. *Muscle Nerve* 1981; 4: 234-245.
- [2] Allen RE, Boxhorn LK: Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor beta, insulin like growth factor I, and fibroblast growth factor. *J Cell Physiol* 1989; 138: 311-315.
- [3] Allen RE, Sheehan SN, Taylor RG, Kendall TG, Rice GM: Hepatocyte growth factor activates quiescent skeletal muscle satellite cells *in vitro*. *J Cell Physiol* 1995; 165: 307-312.
- [4] Austin L, Bower L, Kurek J, Vakakis N: Effects of leukaemia inhibitory factor and other cytokines on murine and human myoblast proliferation. *J Neurol Sci* 1992; 112: 185-191.
- [5] Akira S, Taga T, Kishimoto T: Interleukin-6 in biology and medicine. *Adv Immunol* 1993; 54: 1-78.
- [6] Barnard W, Bower J, Brown MA, Murphy, M, Austin L: Leukemia inhibitory factor (LIF) infusion stimulates skeletal muscle regeneration after injury: injured muscle expressed LIF mRNA. *J Neurol Sci* 1994; 123: 108-113.
- [7] Bartoccioni E, Michaelis D, Hohlfeld R: Constitutive and cytokine-induced production of interleukin-6 by human myoblasts. *Immunol Lett* 1994; 42: 135-138.
- [8] Bischoff R: Analysis of muscle regeneration using single myofibers in culture. *Med Sci Sports Exercise* 1989; 21: 164-172.

MyoD+ cells increase in presence of macrophage cytokines

- [9] Bischoff R: Interaction between satellite cells and skeletal muscle fibres. *Development* 1990; 109: 943-952.
- [10] Bower J, White JD, Kurek JB, Muldoon CM, Austin L: The role of growth factors in myoblast transfer therapy. *Basic Appl Myol* 1997; 7: 177-186.
- [11] Cantini M, Carraro U: Macrophage-released factor stimulates selectively myogenic cells in primary muscle culture. *J Neuropathol Exp Neurol* 1995; 54 : 121-128.
- [12] Cantini M, Massimino ML, Bruson A, Catani C, Dalla Libera L, Carraro U: Macrophages regulate proliferation and differentiation of satellite cells. *Biochem Biophys Res Commun* 1994; 202: 1688-1696.
- [13] Cantini M, Massimino ML, Rapizzi E, Rossini K, Catani C, Dalla Libera L, Carraro U: Human satellite cell proliferation *in vitro* is regulated by autocrine secretion of IL-6 stimulated by soluble factor(s) released by activated monocytes. *Biochem Biophys Res Commun* 1995; 216: 49-53.
- [14] Cantini M, Fiorini E, Catani C, Carraro U: Differential expression of adult type MHC in satellite cell cultures from regenerating fast and slow rat muscle. *Cell Biol Int* 1993; 17: 979-983.
- [15] Chen G, Birnbaum RS, Yablonka-Reuveni Z, Quinn LS: Separation of mouse crushed muscle extracts into distinct mitogenic activities by heparin affinity chromatography. *J Cell Physiol* 1994; 160: 563-572.
- [16] Clegg CH, Linkhart TA, Olwin BB, Hauschka SD: Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. *J Cell Biol* 1987; 105: 949-956.
- [17] Dalla Libera L, Martin B, Massimino ML, Cantini M: Partial purification and biochemical characterization by liquid chromatography of specific myo-mitogenetic factor(s) from culture medium of activated macrophages. *Anal Chim Acta* 1997; 352: 271-275.
- [18] Dijkstra CD, Dopp EA, Joling P, Kraal G: The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2, and ED3. *Immunology* 1985; 54: 89-599.
- [19] Firmia GM, Gottifredi V, Passananti C, Maione R: Double-stranded internucleosomal cleavage of apoptotic DNA is dependent on the degree of differentiation in muscle cells. *J Biol Chem* 1996; 28 : 15575-15579.
- [20] Florini JR, Ewton DZ, Magri KA: Hormones, growth factors and angiogenesis differentiation. *Annu Rev Physiol* 1991; 53: 201-216.
- [21] Florini JR, Magri KA, Ewton DZ, James PL, Grindstaff K, Rotwein PS: "Spontaneous" differentiation of skeletal myoblast is dependent upon autocrine secretion of insulin-like growth factor II. *J Biol Chem* 1991; 266:15917-15923.
- [22] Grounds MD: Towards understanding skeletal muscle regeneration. *Pathol Res Pract* 1991; 118: 1-22.
- [23] Grounds MD, Yablonka-Reuveni Z: Molecular and Cell Biology of skeletal muscle regeneration, in Partridge T (ed): *Molecular and cell biology of muscular dystrophy*. London, Chapman & Hall, 1993, pp 210-256.
- [24] Hannon K, Kudla AJ, McAvoy MJ, Clase KL, Olwin BB: Differentially expressed fibroblast growth factors regulate skeletal muscle development through autocrine and paracrine mechanism. *J Cell Biol* 1996; 132: 1151-1159.
- [25] Honda H, Kimura K, Rostami AM: Demonstration and phenotypic characterization of resident macrophages in rat skeletal muscle. *Immunology* 1990; 70: 272-277.
- [26] Husmann I, Soulet L, Goutron J, Martelly I, Barritoult D: Growth factors in skeletal muscle regeneration. *Cytokine Growth Factor Rev* 1996; 7: 294-258.
- [27] Jones GE, Murphy S J, Wise C, Watt DJ: Macrophages-colony stimulating factor (CSF-1) stimulates proliferation of myogenic cells. *J Cell Biochem Supplement* 1991; 15C: 39.
- [28] Kaufman SJ, Weinstein MG, Faster RF: *In vitro* development of precursor cells in the myogenic lineage. *Dev Biol* 1991; 146: 228-238.
- [29] Laemmli UK: Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 1970; 227: 680.
- [30] Mampuru LJ, Chen S-S, Kalenik JL, Bradley ME, Lee TC: Analysis of the events associated with serum deprivation-induced apoptosis in C3H/Sol8 muscle satellite cells. *Exp Cell Res* 1996; 226: 372-380.
- [31] Massague J: The transforming growth factor- β family. *Annu Rev Cell Biol* 1990; 6: 597-641.
- [32] Massimino ML, Rapizzi E, Cantini M, Dalla Libera L, Mazzoleni P, Arslan P, Carraro U: ED2+ macrophages increase selectively myoblast

MyoD+ cells increase in presence of macrophage cytokines

- proliferation in muscle cultures. *Biochem Biophys Res Comm* 1997; 235: 754-759.
- [33] Mauro A: Satellite cell of skeletal muscle fibres. *J Biophys Biochem Cytol* 1961; 9: 493-495.
- [34] McLennan IS: Resident macrophages (ED2 and ED3-positive) do not phagocytose degenerating rat skeletal muscle fibers. *Cell Tissue Res* 1993; 272: 193-196.
- [35] Merrill CR, Goldman DX, Sedman SA, Erbert MH: Ultrasensitive silver stain for detection proteins in polyacrylamide gels. *Science* 1981; 211: 1437-8.
- [36] Mussini I, Favaro G, Carraro U: Maturation, dystrophic changes and the continuous production of fibers in skeletal muscle regenerating in the absence of nerve. *J Neuropathol Exp Neurol* 1987; 46: 315-331.
- [37] Nathan CF: Secretory products of macrophages. *J Clin Invest* 1987; 79: 319-326.
- [38] Olwin BB, Hauschka SD: Identification of the fibroblast growth factor receptor of Swiss 3T3 cells and mouse skeletal muscle myoblasts. *Biochemistry* 1986; 25: 3487-3492.
- [39] Olson EN: Interplay between proliferation and differentiation within the myogenic lineage. *Dev Biol* 1992; 154: 261-272.
- [40] Olson EN, Sternberg E, Hu JS, Spizz G, Wilcox C: Regulation of myogenic differentiation by type beta Transforming Growth Factor. *J Cell Biol* 1986; 103: 1799-1805.
- [41] Orimo S, Hiyamuta E, Arahata K: Analysis of inflammatory cells and complement C3 in bupivacaine-induced myonecrosis. *Muscle Nerve* 1991; 14: 515-520.
- [42] Quinn LS, Ong LD, Roeder RA: Paracrine control of myoblast proliferation and differentiation by fibroblast. *Dev Biol* 1990; 140: 8-19.
- [43] Robertson TA, Grounds MD, Papadimitriou JM: Elucidation of aspects of murine skeletal muscle regeneration using local and whole body irradiation. *J Anat* 1992; 181: 256-276.
- [44] Robertson TA, Maley MLA, Grounds MD, Papadimitriou JM: The role of macrophages in skeletal muscle regeneration with particular reference to chemotaxis. *Exp Cell Res* 1993; 207: 321-331.
- [45] Sandri M, Massimino ML, Cantini M, Giurisato E, Sandri C, Arslan P, Carraro U: Dystrophin deficient myotubes undergo apoptosis in mouse primary muscle cell culture after DNA damage. *Neurosc Lett* 1998 (in press).
- [46] Schultz E, McCornick KM: Cell biology of the satellite cell, in Partridge T (ed): *Molecular and Cell Biology of Muscular Dystrophy*. London, Chapman & Hall, 1993, pp 190-209.
- [47] Smith J, Schofield PN: The effects of fibroblast growth factors in long term primary culture of dystrophic (MDX) mouse muscle myoblasts. *Exp Cell Res* 1994; 210: 86-93.
- [48] Smith J, Fowkes G, Schofield PN: Programmed cell death in dystrophic (mdx) muscle is inhibited by IGF-II. *Cell Death Diff* 1995; 2: 243-251.
- [49] St Pierre B, Tidball JG: Differential response of macrophage subpopulation to soleus muscle reloading after rat hind limb suspension. *J Appl Physiol* 1994; 77: 290-297.
- [50] Weintraub H, Davis R, Tapscott S, Thayer M, Krause M, Benezra R, Blackwell TK, Turner D, Rupp R, Hollemberg S, Zhuang Y, Lassar A: The MyoD gene family: nodal point during specification of the muscle cell lineage. *Science* 1991; 251: 761-766.
- [51] Weintraub H: The MyoD family and myogenesis: redundancy, networks and thresholds. *Cell* 1993; 75: 1241-1244.
- [52] Yablonka-Reuveni Z, Balestreri TM, Bowen-Pope DF: Regulation of proliferation and differentiation of myoblasts derived from adult mouse skeletal muscle by specific isoforms of PDGF. *J Cell Biol* 1990; 111: 1623-1629.
- [53] Yablonka-Reuveni Z, Seifert RA: Proliferation of chicken myoblasts is regulated by specific isoforms of platelet-derived growth factor: evidence for differences between myoblasts from mid and late stages of embryogenesis. *Dev Biol* 1993; 156: 307-318.