

# Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth

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Once escaped from the quiescence niche, precursor cells interact with stromal components that support their survival, proliferation, and differentiation. We examined interplays between human myogenic precursor cells (mpc) and monocyte/macrophages (MP), the main stromal cell type observed at site of muscle regeneration. mpc selectively and specifically attracted monocytes *in vitro* after their release from quiescence, chemotaxis declining with differentiation. A DNA macroarray-based strategy identified five chemotactic factors accounting for 77% of chemotaxis: MP-derived chemokine, monocyte chemoattractant protein-1, fractalkine, VEGF, and the urokinase

system. MP showed lower constitutive chemotactic activity than mpc, but attracted monocytes much strongly than mpc upon cross-stimulation, suggesting mpc-induced and predominantly MP-supported amplification of monocyte recruitment. Determination of [<sup>3</sup>H]thymidine incorporation, oligosomal DNA levels and annexin-V binding showed that MP stimulate mpc proliferation by soluble factors, and rescue mpc from apoptosis by direct contacts. We conclude that once activated, mpc, which are located close by capillaries, initiate monocyte recruitment and interplay with MP to amplify chemotaxis and enhance muscle growth.

## Introduction

Adult skeletal muscle regeneration results from activation, proliferation, and fusion of myogenic precursor cells (mpc) residing beneath muscle fiber basal lamina, the so-called satellite cells (Hawke and Garry, 2001).

Tissue-specific microenvironmental cues delivered by stromal components influence the fate of both adult stem cells and their progeny (Spradling et al., 2001). The stem cell niche represses differentiation of quiescent and self-renewing cells, whereas the stromal support promotes cell survival and proliferation and appears essential for differentiation of cells escaped from the niche (Spradling et al., 2001). mpc likely depend on such a stromal support to develop their myogenic program (Seale et al., 2001).

Among stromal cells, macrophages (MP) play a major role in maintenance of tissue homeostasis (Gordon, 1995). In addition to phagocytosis and antigen presentation, these cells may play a supportive role through a varied repertoire of plasma membrane and secreted molecules (Gordon, 1995), as shown previously for erythroblasts, hepatocytes, and neurons (Sadahira and Mori, 1999; Takeishi et al., 1999; Polazzi et al., 2001).

Muscle damage induces massive MP infiltration of the injury site (McLennan, 1996; Pimorady-Esfahani et al., 1997). It was shown that irradiation-induced depletion of monocyte/MP impairs muscle regeneration and that reconstitution of bone marrow restores regeneration; moreover, boosted monocyte/MP influx enhances muscle regeneration (Grounds, 1987; Lescaudron et al., 1999). The phagocytic potential of resident MP is debated, but it is generally accepted that newly recruited MP actively remove necrotic debris to

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Abbreviations used in this paper: FKN, fractalkine; HGF, hepatocyte growth factor; HMVEC, human adult microvascular endothelial cells; MCP-1, monocyte chemoattractant protein-1; MDC, MP-derived chemokine; MP, macrophages; mpc, myogenic precursor cells; PBMC, peripheral blood mononuclear cells; uPA, urokinase; uPAR, urokinase type plasminogen-activator receptor.

facilitate subsequent muscle regeneration (McLennan, 1996; Pimorady-Esfahani et al., 1997). In addition, MP likely favor muscle repair as suggested by *in vitro* studies reporting on MP growth-enhancing effects on rodent and avian muscle cells (Robertson et al., 1993; Cantini and Carraro, 1995; Merly et al., 1999). If this view is increasingly acknowledged, the mechanisms by which monocyte/MP are attracted to injury sites and interplay with muscle cells are largely unknown (Tidball et al., 1999). It has been shown previously that peritoneal MP are chemoattracted by crushed murine muscle homogenates but neither the cell source of chemoattractants nor the chemokines involved in this effect were identified (Robertson et al., 1993). Although myoblasts have been occasionally shown to express some chemokine transcripts (De Rossi et al., 2000; Reyes-Reyna and Krolick, 2000), the spectrum of mpc-released chemotactic factors and their role in the recruitment of blood-borne cells have not been delineated. Moreover, enhancement of mpc growth by MP has not been documented in humans and its mechanism remains unknown.

Understanding if and how blood-borne cells interplay with mpc constitute a major goal for developing effective cell therapies of muscle diseases. Moreover, monocyte/MP are already considered as promising therapeutic tools. The fine targeting capacities of MP make possible the use of monocytes as shuttles for gene delivery to hidden or widespread sites of tissue damage, e.g., dystrophic muscle (Parish et al., 1996). In addition, MP can improve natural tissue repair (Rapalino et al., 1998) and, admittedly, recreating an appropriate stromal support in the setting of myoblast transfer therapy could well be most useful to avoid massive mpc death, a major limitation of this therapeutic approach (Skuk and Tremblay, 2000).

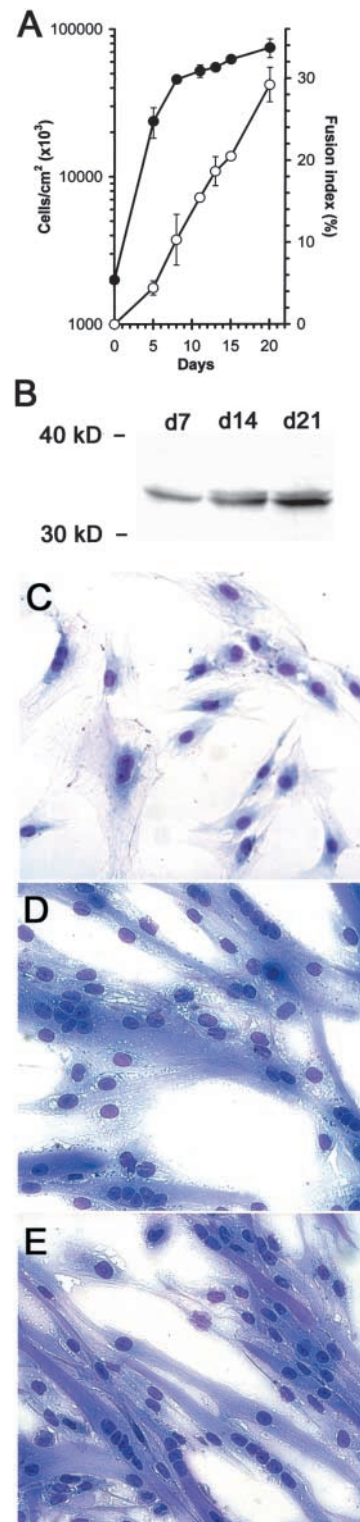
These future prospects prompted us to set up a multistep procedure to determine whether human mpc released from quiescence can initiate monocyte chemotaxis, and to decipher reciprocal influences of mpc and MP that may enhance muscle growth.

## Results

Human mpc in culture were at the stage of proliferation (day 7), early differentiation (day 14), and late differentiation (day 21) when grown without serum withdrawal (Fig. 1). In these conditions, all mpc do not achieve full differentiation. However, myogenesis was assessed by both myotube formation, the fusion index reaching 30% at day 21, and increasing myogenin expression (Fig. 1).

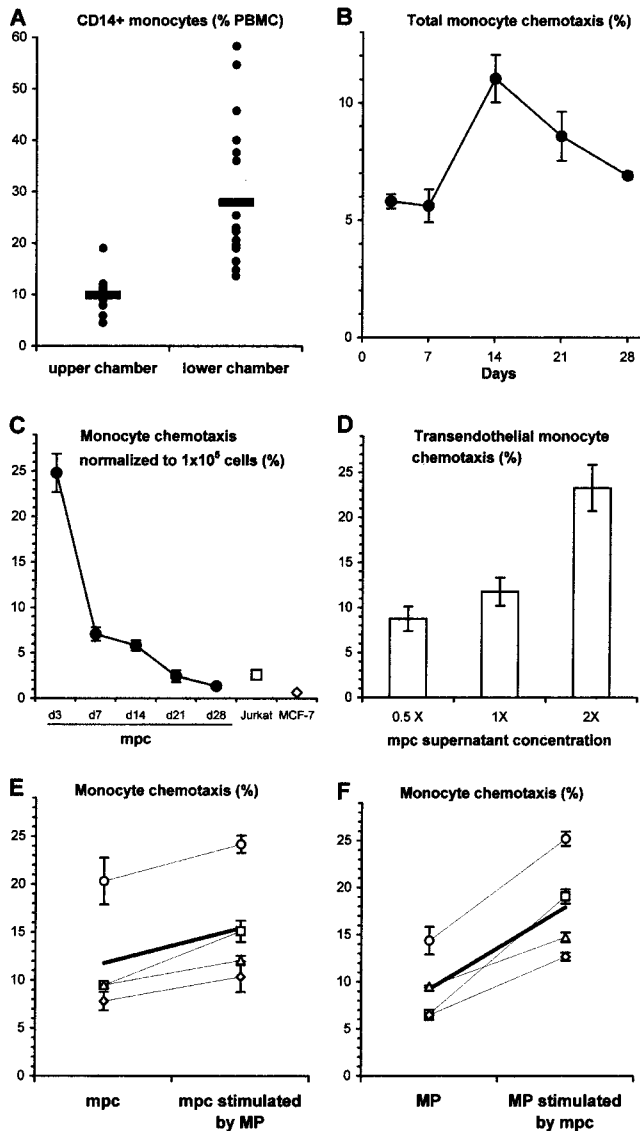
### Constitutive monocyte chemotaxis by mpc

**Human mpc attract monocytes.** In conventional chemotaxis assays, human mpc attracted peripheral blood mononuclear cells (PBMC), 5% of PBMC being specifically attracted at day 7, 9% at day 14, and 6% at day 21 (day 7 vs. 14 increase,  $P < 0.003$ ; day 14 vs. 21 decrease,  $P < 0.05$ ). Chemotaxis selectively involved monocytes as assessed by enrichment of the attracted cells in CD14<sup>+</sup> cells (28% vs. 10%,  $P < 0.0001$ ; Fig. 2 A). Enrichment in CD14<sup>+</sup> cells was similar at all stages of mpc culture and was not due to modulation of CD14 expression by the mpc-conditioned medium (unpublished data).



**Figure 1. In vitro human mpc myogenesis.** (A) mpc growth is expressed in number of cells/cm<sup>2</sup> (closed circles) and mpc differentiation is estimated by the fusion index (open circles). (B) Myogenin immunoblot at days 7, 14, and 21 of mpc culture. (C–E) May-Grünwald Giemsa stain of mpc at days 7 (C), 14 (D), and 21 (E) of culture.

Similarly to PBMC, isolated human monocytes were attracted by mpc with a peak of chemotaxis at day 14 (day 7 vs. 14 increase,  $P < 0.0001$ ; day 14 vs. 21 decrease,  $P < 0.005$ ; Fig. 2 B). Because chemotactic activity of a differenti-



**Figure 2. Monocyte chemotaxis by mpc is specific, is regulated during myogenesis and is enhanced by mpc/MP interplay.** (A) Percentage of CD14<sup>+</sup> cells among PBMC before (upper chamber) and after (lower chamber) chemotaxis toward mpc-conditioned medium. Each circle represents one experiment and bars are the means. (B) Monocyte chemotaxis toward mpc-conditioned medium during myogenesis. (C) Monocyte chemotaxis normalized to  $1 \times 10^5$  cells. (D) Monocyte chemotaxis toward mpc-conditioned medium (day 14) across HMVEC monolayer. All results in (A–D) are means  $\pm$  SEM of at least three experiments run in triplicate. (E and F) Monocyte chemotaxis by (E) mpc (day 14) and (F) MP stimulated or not by the other cell type. Each symbol represents one experiment run in triplicate and variations are SD. Thick bar represents the mean.

ating cell population may reflect both the state of differentiation and the number of cells at each time point, we calculated chemotaxis normalized for  $10^5$  mpc at each time point. Fig. 2 C shows that normalized mpc chemotactic activity was high at day 3, dropped at day 7, and progressively declined at subsequent stages of differentiation. Differentiated myotubes exhibited a low normalized chemotactic activity similar to that of other cell types, including Jurkat and MCF-7 cells. In standard culture conditions, the volume of

medium remains constant at each time point. To avoid bias in calculation of normalized mpc chemotactic activity due to variations of medium volume/cell number ratio, we measured chemotaxis at a constant ratio. Days 3 and 7 time points exhibited the highest difference of volume/cell number ratio in standard conditions. Chemotaxis measured at a constant ratio showed a decrease by 42% of mpc chemotaxis from days 3 to 7 ( $P < 0.005$ ; unpublished data), confirming that mpc exhibit maximal individual chemotactic activity shortly after their release from quiescence. A similar experiment conducted at days 7 and 14 revealed a decline of chemotaxis by 18% (unpublished data), very close to that obtained by calculation (19%).

Normalized chemotaxis of mpc grown in differentiating conditions to stimulate myotube formation showed a more abrupt decrease than that of mpc allowed to spontaneously differentiate (unpublished data), confirming that mpc differentiation is associated with a decline of their chemotactic activity.

We assessed that monocyte attraction was directional by varying mpc-conditioned medium concentrations in the chemotaxis chambers. Increasing gradients from the upper to the lower chamber induced migration of monocytes, but neither absent nor reverse gradients did; moreover, chemotaxis correlated positively with the gradient magnitude (unpublished data).

We used microvessel-derived endothelial cells to control that mpc chemotaxis remains operative across an endothelial layer. Using various mpc supernatant concentrations, we observed a dose-dependent transendothelial monocyte migration ( $P < 0.05$ ; Fig. 2 D). This assay approximated the *in vivo* situation as demonstrated by microanatomic study of human adult muscle. Indeed, a majority of CD56<sup>+</sup> satellite cells were located close by capillaries (87% being 5–20  $\mu\text{m}$  from a capillary). The mean distance from a satellite cell nucleus to the closest capillary lumen center was  $12.7 \pm 7.5 \mu\text{m}$ .

**DNA array yields a set of candidate chemotactic factors in mpc.** Instead of studying a preselected set of molecules to identify the mpc-produced effectors of monocyte chemotaxis, we used an mRNA profiling technique allowing analysis of a huge number of genes at once. Among the 588 genes represented on the DNA macroarray membrane we used, 20 had products known to attract monocytes, of which five were constitutively expressed by human mpc at days 7 and 14 of culture (Table I): monocyte chemoattractant protein-1 (MCP-1, CCL2; Zachariae et al., 1998), MP-derived chemokine (MDC, CCL22; Mantovani et al., 2000), fractalkine (FKN, CX<sub>3</sub>CL1; Bazan et al., 1997), VEGF (Sawano et al., 2001), and urokinase type plasminogen-activator receptor (uPAR; Resnati et al., 1996).

Confirmatory RT-PCR showed amplification products of MCP-1, MDC, FKN, and VEGF transcripts at the expected molecular weight (Fig. 3 A) in mpc culture. Both expression and up-regulation of a functional uPAR/urokinase (uPA) chemokine-like system during human mpc differentiation were reported previously (Quax et al., 1992; Chazaud et al., 2000). Constitutive mpc release of MCP-1, MDC, and VEGF was assessed by ELISA. MDC level normalized for  $1 \times 10^5$  cells was higher at day 3 than at day 7 ( $P < 0.001$ ), and at day 7

Table I. Gene expression by mpc at days 7 and 14 of culture

Gene name	GenBank accession no.	Intensity <sup>a</sup>	
		Day 7	Day 14
Eotaxin (CCL11)	D49372; Z75669; Z75668	–	–
Fractalkine (CX <sub>3</sub> CL1)	U91835	35	35
GCSF (granulocyte colony–stimulating factor)	X03438	–	–
I309 (CCL1)	M57502	–	–
IL8 (interleukin-8) (CXCL8)	Y00787	–	–
IP10 (interferon $\gamma$ -induced protein) (CXCL10)	X02530	–	–
LT- $\alpha$ (lymphotoxin- $\alpha$ )	D12614	–	–
MCP-1 (monocyte chemotactic protein 1) (CCL2)	M24545	51	166
MCP-2 (CCL8)	Y10802	–	–
MCP-3 (CCL7)	X72308	–	–
MDC (CCL22)	U83171	30	29
MIP1- $\alpha$ (macrophage inflammatory protein 1 $\alpha$ ) (CCL3)	M23452	–	–
MIP1- $\beta$ (CCL4)	J04130	–	–
MIP3- $\beta$ (CCL19)	U77180	–	–
PDGFBB (B)	X02811; M12783; M16288	–	–
RANTES (CCL5)	M21121	–	–
TNF- $\alpha$	X01394	–	–
uPAR (CD87)	U08839; M83246; X51675	7	7
VEGF C	U43142	73	74

<sup>a</sup>Measured in arbitrary units.

than at day 14 ( $P < 0.05$ ; Fig. 3 B). MCP-1 was undetectable at day 3, appeared at day 7, and abruptly increased from days 7 to 14 ( $P < 0.02$ ; Fig. 3 B). Consistently, RT-PCR did not detect MCP-1 transcripts at day 3 (not depicted), and DNA microarray showed a threefold increase of MCP-1 mRNA level from days 7 to 14 (Table I). VEGF level variations exhibited a strong increase at day 21 ( $P < 0.005$ ; Fig. 3 C). Soluble FKN levels did not reach the high detection threshold (70 pg/ml) of the ELISA we used (Foussat et al., 2001). However, immunofluorescence confirmed cellular expression of FKN and the three other chemokines (Fig. 3, D–G). Labeling of multinucleated cells unequivocally assessed a myogenic cell expression. Cytoplasmic immunopositivity was observed for all molecules. In addition, marked cell membrane labeling was observed for FKN (Fig. 3 D).

**MCP-1, MDC, FKN, VEGF, and the uPAR/uPA system are the main effectors of monocyte chemotaxis by mpc.** Functional involvement of the detected molecules was assessed using specific blocking antibodies (Fig. 4). Inhibition tests could not be carried out at day 3, the time point associated with the highest individual mpc chemotactic activity, because of a too low cell number in the conditions we used and impracticability of increasing cell density without influencing myogenic differentiation. Therefore, inhibition tests were performed at day 14, a time point associated with the highest global mpc chemotactic activity. Monocyte chemotaxis decreased by 45% after MCP-1 inhibition ( $P < 0.01$ ), 50% after MDC inhibition ( $P < 0.005$ ), 62% after FKN inhibition ( $P = 0.003$ ), 44% after VEGF inhibition ( $P < 0.02$ ), and 26% after uPAR inhibition ( $P < 0.02$ ). Whole Igs induced no effect. The presence of soluble FKN was assessed by blocking the cognate receptor CX<sub>3</sub>CR1 on monocytes, which inhibited chemotaxis by 59% ( $P < 0.005$ ). To further inhibit the complicated mechanism underlying the

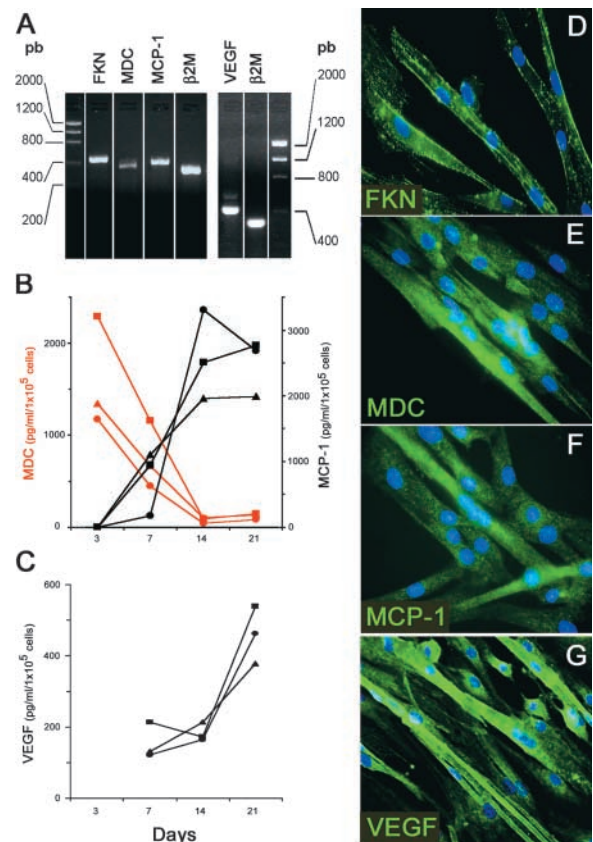
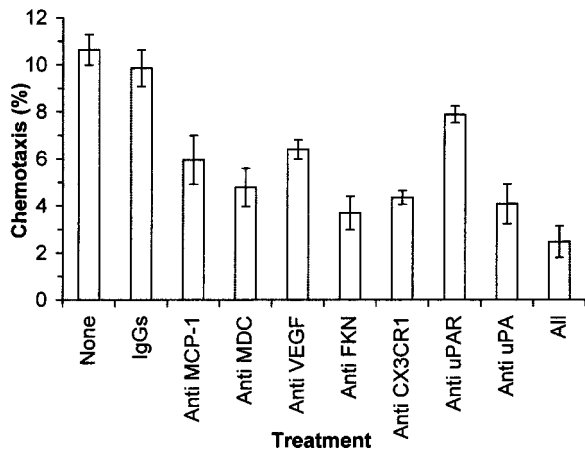


Figure 3. **mpc constitutively express 5 monocyte chemotactic factors.** (A) RT-PCR analysis of mpc mRNA at day 14 of FKN, MDC, MCP-1, VEGF, and  $\beta$ 2M ( $\beta$ 2microglobulin). (B and C) ELISA for (B) MDC, MCP-1, and (C) VEGF of mpc supernatants. Each symbol represents one culture estimated in triplicate. (D–G) Immunolabeling of (D) FKN, (E) MDC, (F) MCP-1, (G) VEGF using FITC-conjugated secondary antibody. Blue, DAPI stain.



**Figure 4. Five chemotactic systems ensure monocyte chemotaxis by mpc.** Monocyte chemotaxis toward mpc-conditioned medium (day 14) was performed in the absence or presence of whole mice and rabbit IgGs or antibodies directed against MCP-1, MDC, VEGF, FKN, CX<sub>3</sub>CR1, uPAR, and uPA. Results are the means  $\pm$  SEM of three experiments run in triplicate.

uPAR/uPA chemokine-like effect we targeted uPA, a strategy previously proved efficient (Resnati et al., 1996). uPA inhibition induced a 58% decrease of chemotaxis ( $P < 0.003$ ). Because leukocytes integrate the various chemoattractant signals they receive through multiple and promiscuous receptors in a complex and still poorly understood fashion (Foxman et al., 1997), we analyzed the effect of global effector inhibition. Pooling blocking antibodies against MCP-1, MDC, FKN, VEGF, uPAR, and uPA induced a 77% inhibition of monocyte chemotaxis ( $P < 0.03$ ; Fig. 4).

**Monocyte chemoattractants are expressed by activated satellite cells in vivo.** In vivo relevance of our findings was assessed by double immunostaining for CD56 and each ef-

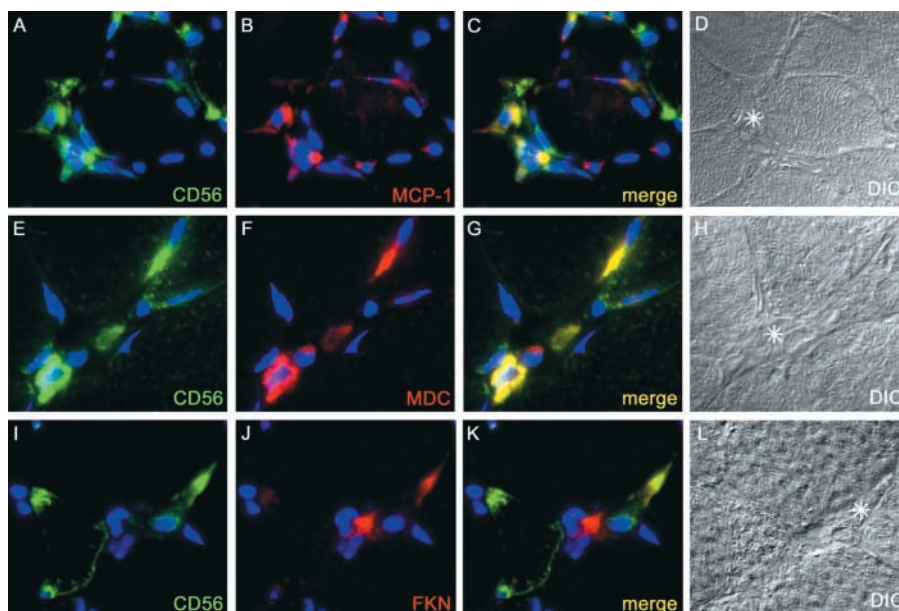
factor on cryosections of a muscle biopsy showing pure necrotizing myopathy, i.e., patchy degeneration/regeneration without lymphocytic infiltrates. Coexpression of CD56 and either MCP-1, MDC, or FKN was restricted to mononucleated cells located in foci of postnecrotic regeneration (Fig. 5). Because expression of chemoattractants was not observed in normal looking muscle areas, we assume that these CD56-positive cells corresponded to either activated satellite cells or myoblasts. In addition, immunopositivities were observed in nonmyogenic mononuclear cells, presumably MP, within or close to diseased areas (Fig. 5).

### mpc/MP interplays

**mpc and MP interact to amplify monocyte chemotaxis.** As compared to classically cultured mpc, mpc incubated 30 h with MP-conditioned medium increased by 31% of their chemotactic effect on monocytes ( $P < 0.02$ ; Fig. 2 E). The factors involved in constitutive mpc chemotaxis were also implicated here because global inhibition decreased monocyte chemotaxis by 67% ( $P < 0.006$ ; unpublished data). MP stimulation of mpc chemotaxis was specific because it was not reproduced by mpc-conditioned medium ( $P = 0.62$ ; unpublished data). Conversely, MP incubated with mpc-conditioned medium increased their chemotaxis by 94% ( $P < 0.02$ ; Fig. 2 F). This stimulation was not reproduced by MP-conditioned medium ( $P = 0.80$ ; unpublished data).

**MP stimulate mpc growth.** Cocultures at various mpc/MP ratios were performed to further evaluate cell interplays. First, we examined if MP operate phagocytosis of PKH26-labeled mpc. No intracytoplasmic fluorescent signal was observed in MP after 1–4 d of coculture, whatever the cell ratio (ranging from 1:0.5 to 1:2), ruling out significant phagocytosis of living mpc by MP (Fig. 6, A and B).

Next, mpc growth curves were established under culture conditions allowing, or not, direct mpc/MP contacts. MP



**Figure 5. Activated satellite cells express the monocyte chemoattractants in vivo.** Muscle biopsy from a patient with pure necrotizing myopathy was labeled for both chemoattractants (red) and CD56, a marker of myogenic cells, including satellite cells, myoblasts and regenerating muscle fibers (green). Colocalization (yellow in merging pictures) was observed in mononucleated cells within diseased areas (star in differential interference contrast (DIC) pictures) including shrunk muscle fibers undergoing postnecrotic regeneration. As usual in pathological muscle, the degenerative process and regeneration take place almost simultaneously within the same fiber. (A–D) Several mononucleated myogenic cells and two neighboring nonmyogenic cells express MCP-1. (E–H) Three myogenic cells and one neighboring nonmyogenic cell express MDC, whereas one regenerating fiber does not (top right). (I–L) One myogenic cell and one neighboring nonmyogenic cell (CD56-) express FKN, whereas another regenerating fiber does not (bottom left). Blue, DAPI stain.

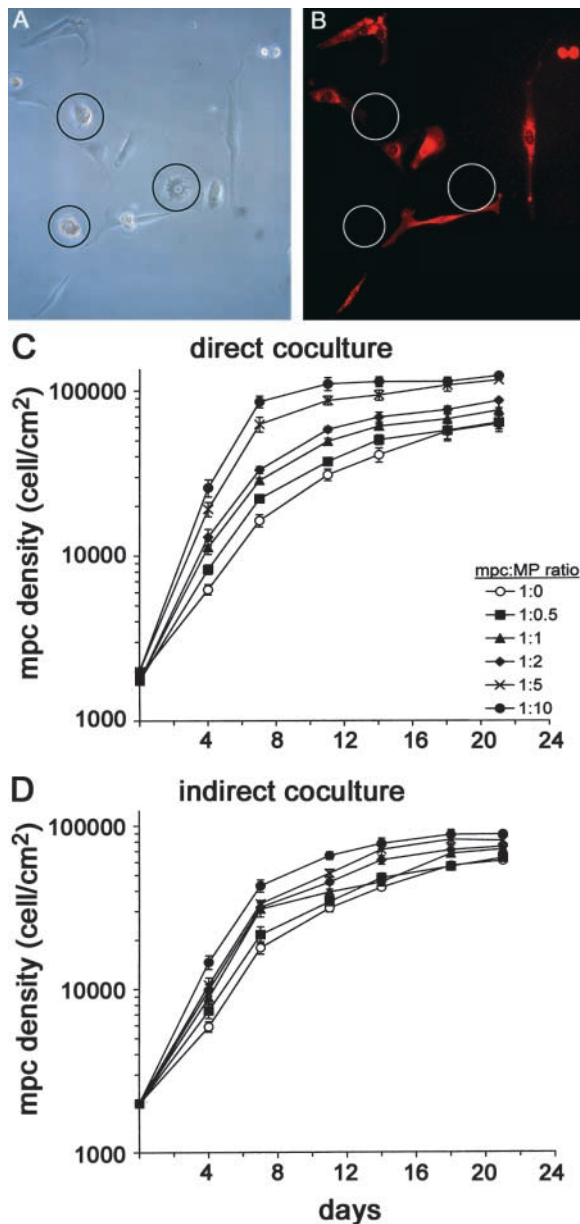


Figure 6. **mpc/MP cocultures stimulate mpc growth.** (A and B) coculture of PKH26-labeled mpc with MP (1:1 ratio) for 2 d shows absence of fluorescence in MP cytoplasm (circles). (C and D) Density of mpc in (C) direct or (D) indirect cocultures with MP at various seeding ratio. Results are the means  $\pm$  SEM of three experiments run in duplicate.

induced a dose-dependent increase of mpc density in both conditions, but enhancement was stronger in conditions allowing mpc/MP contacts (Fig. 6 C) than in cultures separated by a porous filter (Fig. 6 D; 5.3-fold vs. 2.4-fold increase of mpc density at day 7 of culture at the 1:10 [mpc/MP] ratio,  $P < 0.02$ ).

**MP promote mpc proliferation by soluble factors and mpc survival by direct contacts.** mpc proliferation, quantified by [ $^3$ H]thymidine incorporation, was strongly promoted by MP-conditioned medium in a dose-dependent way, an increase of 126% being observed at the 1:2 (mpc/MP) ratio ( $P < 0.004$ ; Fig. 7 A). mpc proliferation could be specifically evaluated in cocultures because human MP are

postmitotic cells (van der Meer et al., 1982) that do not incorporate [ $^3$ H]thymidine (Fig. 7 B). mpc proliferation was moderately decreased by direct contact with MP, a decrease of 27% being observed at the 1:2 (mpc/MP) ratio ( $P < 0.004$ ; Fig. 7 B). Therefore, the net cell growth increase observed in cocultures allowing cell–cell contacts could not be attributed to a mitogenic effect. Rescuing of mpc from apoptosis being linked to cell cycle exit (Dee et al., 2002), an antiapoptotic effect mediated by MP contacts was likely. This was confirmed by determination of oligosomal DNA levels showing much lower apoptosis in cocultures (1:1 ratio) than expected from addition of the levels determined in separated mpc and MP cultures (Fig. 8 A). To discriminate between variations of apoptosis affecting mpc and MP, we performed double labeling with anti-CD56 antibody, a mpc marker, and annexin-V, an early marker of apoptosis. As compared with separate cultures, cocultures at 1:1 ratio showed a decreased number of both apoptotic mpc (annexin-V $^+$ , CD56 $^+$  cells; 48.1 vs. 17.3%,  $P < 0.02$ ) and apoptotic MP (annexin-V $^+$ , CD56 $^-$  cells; 63.1 vs. 39.9%,  $P < 0.01$ ; Fig. 8, B–D). Rescuing of mpc from death could not be attributed to soluble factors because mpc apoptosis remained unchanged upon administration of MP-conditioned medium (Fig. 8 A).

## Discussion

We showed herein that human mpc could selectively and specifically attract monocytes through an endothelial layer in a dose-dependent fashion. This previously unreported property of mpc varied according to the differentiation stage, individual chemotactic activity of satellite cells being high shortly after their release from quiescence and then declining progressively to reach levels similar to that of other cell types at time of late differentiation into multinucleated myotubes. mpc were shown to produce five monocyte chemoattractants accounting for 77% of whole mpc chemotaxis at day 14 of culture. They included three chemokines, MDC, MCP-1, and FKN, one growth factor, VEGF, and one proteolytic system with chemotactic activity, uPA/uPAR. Effectors exhibited clearly different secretion profiles. MDC, a recently identified CC-chemokine, was the earliest detected signal for monocyte recruitment. MCP-1, a more extensively studied CC-chemokine, took over from MDC at time of later stages of myogenic differentiation.

MDC is not detected in normal human adult skeletal muscle (Mantovani et al., 2000). It functions through the CCR4 receptor, which is expressed by 6% of human monocytes (Katschke et al., 2001), and at least another important, as yet unknown, receptor (Mantovani et al., 2000). In addition to its chemotactic effect on monocytes, MDC activates MP and enhances their phagocytic activity more rapidly than does MCP-1, *in vivo* (Matsukawa et al., 2000).

MCP-1 is produced, mainly under proinflammatory conditions, by a large variety of cells (Zachariae et al., 1998). CCR2 receptor, that is expressed by 71% of human monocytes (Fantuzzi et al., 1999), mediates MCP-1 effects on monocyte chemotaxis and activation (Zachariae et al., 1998). Constitutive myogenic cell expression of MCP-1 was reported previously in rat (Reyes-Reyna and Krolick, 2000)

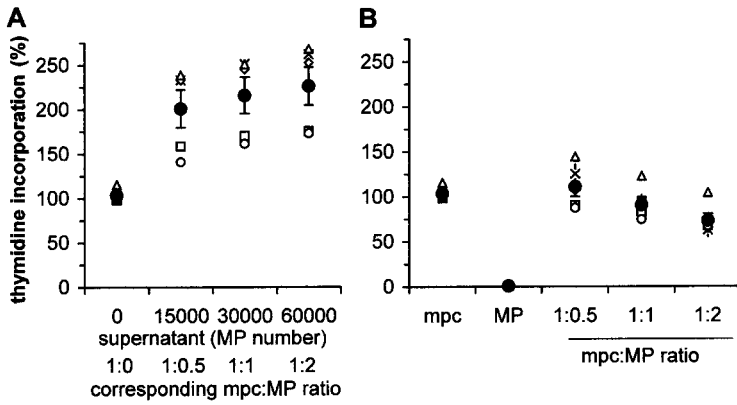


Figure 7. **MP-secreted factors enhance mpc proliferation.** [<sup>3</sup>H]thymidine incorporation of mpc treated with (A) MP-conditioned medium or (B) cocultured with MP. Each open symbol represents one separate experiment run in triplicate and closed circles represent the means ± SEM.

and human rhabdomyosarcoma (Astolfi et al., 2001) cell lines, but not in primary human mpc cultures (De Rossi et al., 2000). Interestingly, we did not detect MCP-1 at day 3, a very early stage of culture, although a unique up-regulation of MCP-1 mRNA expression was documented at subsequent stages. Such a differentiation-associated up-regulation

of MCP-1 expression was reported previously in both a rhabdomyosarcoma cell line (Astolfi et al., 2001) and monocytes/MP (Fantuzzi et al., 1999). Up-regulation of MCP-1 production is pivotal for amplification of chemotaxis (Andjelkovic et al., 2000). Thus, MCP-1 appears as a secondary signal for monocyte recruitment and MP activation, deliv-

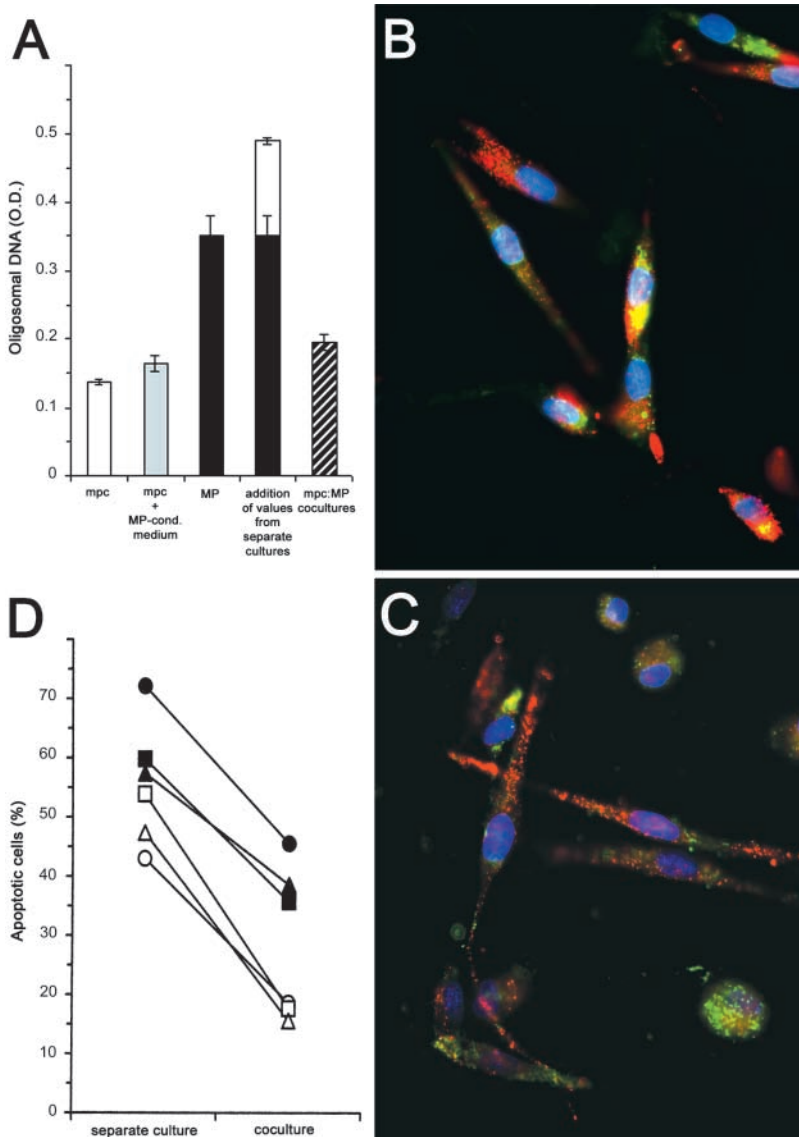


Figure 8. **MP rescue mpc from apoptosis.** (A) Oligosomal DNA measurement in mpc cultures, mpc treated with MP-conditioned medium, MP, and mpc/MP cocultures. Results are the means ± SD of three experiments run in duplicate. (B–D) mpc were (B) cultured alone or with (C) MP and labeled with annexin-V (green) and anti-CD56 antibodies (red). (B and C) Blue, DAPI stain. (D) Quantification of apoptotic cells among MP (black symbols) and mpc (white symbols) populations. Each symbol represents one separate culture.

ered by mpc at time of MDC down-regulation in the setting of chemotaxis amplification.

VEGF induces vascular cell chemotaxis, survival, and proliferation, mainly through VEGF-R2 (Rissanen et al., 2002). Among its nonvascular roles, VEGF is chemotactic for monocytes through VEGF-R1, a receptor expressed by 83% of human monocytes (Sawano et al., 2001). Muscle fiber expression of VEGF and VEGF-R2 is induced by ischemia (Rissanen et al., 2002). It is associated with focal MP infiltration and vessel hyperplasia and might prevent muscle cell death and support regeneration (Rissanen et al., 2002). Similar VEGF effects may be at play after other types of muscle injury as well.

The CX<sub>3</sub>C chemokine FKN contains a chemokine domain fused to a mucin-stalk tethered to a transmembrane domain with an intracytoplasmic tail (Bazan et al., 1997). FKN transcripts have been detected previously in normal human muscle homogenates (Bazan et al., 1997). In FKN-producing cells, such as endothelial cells, 90% of FKN is membrane bound at steady state and 10% is cleaved in a soluble form (Imaizumi et al., 2000). Soluble FKN is angiogenic (Volin et al., 2001) and chemotactic for monocytes (Bazan et al., 1997; Chapman et al., 2000) through the cognate receptor CX<sub>3</sub>CR1, that is expressed by 56% of human monocytes (Ruth et al., 2001). In our work, both anti-FKN and anti-CX<sub>3</sub>CR1 antibodies inhibited mpc chemotactic activity but FKN could not be detected in supernatants by ELISA. This was in keeping with previous evidence that attraction of human monocytes by FKN may occur at concentrations far below the ELISA detection threshold (Chapman et al., 2000).

The uPA system mainly includes the receptor uPAR, its ligand uPA and the matrix-bound inhibitor PAI-1 (Preissner et al., 2000). The three components are markedly up-regulated during muscle regeneration (Festoff et al., 1994; Lluís et al., 2001) and at time of fusion in human mpc cultures (Quax et al., 1992; Chazaud et al., 2000). uPA activates hepatocyte growth factor (HGF) through cleavage of its matrix-associated inactive precursor, which might trigger activation of quiescent satellite cells through c-met, the HGF receptor (Allen et al., 1995). In addition, the uPA system exerts proteolytic and nonproteolytic roles operative in cell migration (Chazaud et al., 2000; Preissner et al., 2000). A soluble form of truncated uPAR, present in body fluids (Sidenius et al., 2000), mediates chemotaxis of myelomonocytic cells by inducing signal transduction through an unknown transmembrane adaptor (Resnati et al., 1996). uPA exerts similar chemotactic effects through uPAR and the same unknown adaptor (Resnati et al., 1996). In our system, uPAR blockade could not assess the proper role of soluble uPAR because it interfered with uPA–uPAR binding at the membrane of monocytes. Consistently, anti-uPA antibodies induced inhibition of chemotaxis. A crucial role of uPA in muscle regeneration was demonstrated in uPA deficient mice (Lluís et al., 2001), and reflects the multifunctional status of the uPA system that could control satellite cell activation, monocyte chemotaxis and mpc migration (Chazaud et al., 2000).

Finally, attempts to delineate the respective contribution of each chemoattractant to chemotaxis exerted by a conditioned medium containing multiple effectors constitute a somewhat vain objective as: (a) chemotaxis results from the

integration of stimulating, inhibiting, and nullification signals converging on a common chemotaxis-initiating pathway (Foxman et al., 1999); (b) as a result of receptor saturation, homologous desensitization, or receptor sequestration, chemoattractant concentration is not linearly correlated to the observed chemotaxis (Foxman et al., 1997); and (c) chemokines may be inactivated upon cleavage by MMPs at the NH<sub>2</sub> terminus (McQuibban et al., 2002). For example, truncated forms of MCP-1, that only lack two or three amino acids and are likely detected by ELISA, were likely present in our model that is associated with MMP up-regulation (Guerin and Holland, 1995).

We showed that mpc and MP cross-stimulate their chemotactic activity through soluble factors. Other cell types were shown previously to interplay with monocyte/MP to enhance MCP-1 production (Andjelkovic et al., 2000). The activating factors remain undetermined, but activated muscle cells are known to produce cytokines, including interleukin-1 (Authier et al., 1999), interleukin-6 (Cantini et al., 1995), TNF $\alpha$  (Saghizadeh et al., 1996), and MCP-1 (De Rossi et al., 2000), that strongly up-regulate MCP-1 expression by monocyte/MP (Zachariae et al., 1998). Constitutively, MP exerted a lower chemotactic activity than mpc, but they amplified this activity more strongly than mpc did upon reciprocal stimulation. This suggests that, once recruited to the injured area, mpc-conditioned MP may progressively substitute for mpc to recruit additional monocytes. In addition, MP also attract myogenic cells (Robertson et al., 1993), which may favor their migration along muscle fibers or even across the basal lamina toward the site of postinjury regeneration (Carpenter and Karpati, 2001). Immunohistochemical study of a human necrotizing myopathy confirmed *in vivo* expression of chemoattractants by activated satellite cells and some regenerating muscle fibers. In contrast, muscle fibers either intact or at an early necrotic stage showed no expression of chemoattractants. In addition to satellite cells, there were nonmyogenic mononuclear cells expressing chemoattractants in areas of muscle regeneration, presumably including MP, as reported previously in the setting of inflammatory myopathies (Liprandi et al., 1999; Confalonieri et al., 2000).

MP were also shown to stimulate human mpc growth. MP-produced soluble factors promoted mpc proliferation. A number of such factors have been identified, including IGF I and II, HGF, FGFs, PDGF<sup>BB</sup>, EGF, and interleukin-6 (Robertson et al., 1993; Hawke and Garry, 2001). In addition, MP contacts rescued a number of mpc from apoptosis. Up to 35% of myoblasts die by apoptosis upon growth factor deprivation, whereas the others rapidly differentiate into syncytial cells, a cell type more resistant to apoptosis (Dee et al., 2002). Environmental factors that may rescue mpc from apoptosis are unknown. Our results strongly suggest involvement of adhesion molecules. Other cell types, including erythroblasts, astrocytes, and neurons, may be rescued by MP through VLA-4 ( $\alpha_4\beta_1$  integrin)–VCAM-1 or FKN–CX<sub>3</sub>CR1 interactions (Sadahira and Mori, 1999; Meucci et al., 2000). These molecules are expressed by both mpc and MP, as reported previously (Rosen et al., 1992; Sadahira and Mori, 1999; Meucci et al., 2000), or assessed by DNA microarray (CX<sub>3</sub>CR1; unpublished data). Here, we showed con-



spicuous labeling of membrane-bound FKN by mpc. As other cell types, mpc might use FKN–CX<sub>3</sub>CR1 interactions to bind MP (Fong et al., 1998) and deliver or receive antiapoptotic signals (Meucci et al., 2000). In the same way, binding of recruited leukocytes by satellite cells and newly formed myotubes involving VLA-4–VCAM-1 interactions was documented previously *in vitro* and *in vivo* (Jesse et al., 1998) and blockade of  $\beta_1$  integrin causes mpc apoptosis (Vachon et al., 1997).

We propose a model in which, shortly after their release from quiescence after muscle injury or the more limited exercise-induced myotrauma causing adaptive muscle hypertrophy (Hawke and Garry, 2001), satellite cells, either alone or in combination with other resident cells types, initiate a process aimed at providing an adequate stromal support to their growth. The process first consists in mpc attraction of circulating monocytes, which is facilitated by their remarkable proximity to muscle capillaries (Schmalbruch and Hellhammer, 1977). Although differentiating into MP, the blood-borne cells interplay with mpc to amplify the chemotactic activity of the injured muscle area, and progressively become the main local source of chemoattractants. At the same time, MP phagocytose dead cells and/or act as potent supportive cells for living mpc through delivery of both soluble mitogenic factors and cell contact-mediated survival signals, ensuring good repair or maintenance of skeletal muscle.

This paper provides further support for therapeutic strategies using monocytes as shuttles to treat muscular dystrophies by the vascular route (Parrish et al., 1996). Moreover, it is very unlikely that mpc transplanted into muscle for therapeutic purposes would efficiently orchestrate the fine setting up of the stromal support they need to grow and differentiate *in vivo*, as many as 95% of transplanted mpc die shortly after injection (Skuk and Tremblay, 2000) for nonmechanical causes (Chazaud et al., 2003). Our results open the possibility of improving myoblast transfer therapy by providing transplanted mpc with appropriate environmental cues.

## Materials and methods

### Cell cultures

Unless indicated, culture media components were obtained from GIBCO BRL and culture plastics were obtained from TPP AG.

Human mpc were cultured from muscle samples as described previously (Chazaud et al., 2000). In standard conditions (spontaneous *in vitro* myogenesis), mpc were grown in Ham's F12 medium containing 15% FCS (growing medium) without serum withdrawal. In differentiating conditions, growing medium was replaced by Ham's F12 medium containing 5% FCS (differentiating medium) at time of subconfluence. Only cultures presenting over 95% CD56+ (1:20 dilution, 123C3 clone; Sanbio/Monosan) cells were used.

PBMC were isolated from human blood using Ficoll Paque plus (Amersham Biosciences) density gradient. Monocytes were isolated from PBMC by an adhesion step (Fantuzzi et al., 1999). Purity, estimated by flow cytometry after CD45-FITC (KC56 clone; Beckman Coulter) and CD14-PE (RMO52 clone; Immunotech) labeling, ranged from 80 to 90%.

To obtain MP, monocytes were seeded at  $0.5 \times 10^6$  cell/ml in Teflon bags (AFC) in differentiating RPMI medium containing 15% human AB serum for 8 d (van der Meer et al., 1982).

Jurkat cells were grown in RPMI containing 10% FCS. MCF-7 cells were grown in DME containing 5% FCS and 1% nonessential amino acids.

mpc/MP coculture ratio ranged from 1:0.5 to 1:10. For growth and immunolabeling experiments,  $1 = 2,000$  cells/cm<sup>2</sup>. In indirect cocultures, MP were seeded in inserts (0.4- $\mu$ m diam pores; Falcon™, BD Biosciences) placed over the mpc-containing well. In 96-well plates (proliferation and apoptosis assays),  $1 = 30,000$  cells/well.

### Conditioned media

Conditioned media were obtained by incubating cells in 24-well plates in serum-free Ham's F12 for 24 h in 0.5 ml (1 $\times$ ). For chemotaxis assay at constant cell/supernatant ratio, the volume of supernatant was adjusted exactly to the mpc number (300  $\mu$ l for 10,000 mpc). Two and three times the concentrations of mpc-conditioned media were obtained by proportional reduction of medium in mpc culture. Cell stimulation by conditioned medium was performed by incubating cells for 30 h with medium conditioned the day before.

### mpc growth and differentiation

mpc density was determined by counting cells after trypsinization. Trypsin treatment did not detach MP, as flow cytometry analysis of detached cells after CD64-FITC (10.1; BD Biosciences) and CD14-PE labeling showed no CD14+ and <0.4% CD64+ cells. Fusion index was calculated as described previously (Authier et al., 1999). Myogenin immunoblotting was carried out using 40  $\mu$ g mpc protein extract and M-225 antibody (1:200; Santa-Cruz Biotechnology, Inc.).

### Chemotaxis

Leukocytes (500,000 in serum-free Ham's F12 medium) were deposited into Falcon insert (3- $\mu$ m diam pores) put on top of a well containing conditioned medium and plates were incubated at 37°C for 24 h. The number of cells present in the well was evaluated and expressed as percentage of number of deposited cells. Chemotaxis toward Ham's F12 medium was considered as nonspecific chemotaxis, which value was subtracted from observed values. No leukocyte was present at the insert lower face. In some experiments, blocking antibodies were added in the well at saturating concentrations (calculated from IC50 or from previous studies): 3  $\mu$ g/ml anti-MCP-1 (Abcys), 3  $\mu$ g/ml anti-FKN (51637.11 clone; R&D Systems), 6  $\mu$ g/ml anti-MDC (R&D Systems), 6  $\mu$ g/ml anti-VEGF (R&D Systems), 15  $\mu$ g/ml anti-CX<sub>3</sub>CR1 (Torrey Pines Biolaboratories; Chapman et al., 2000), 4  $\mu$ g/ml anti-uPA (American Diagnostica, Inc.), 5  $\mu$ g/ml anti-uPAR (American Diagnostica, Inc.; Chazaud et al., 2002). Controls included addition of whole mice and rabbit IgGs (3  $\mu$ g/ml each; Vector Laboratories).

Transendothelial chemotaxis was performed using human adult microvascular endothelial cells (HMVEC) cultured according to the manufacturer's instructions (Biowhittaker). HMVEC were seeded at 10,000 cells/cm<sup>2</sup> in Falcon inserts (3- $\mu$ m diam pores). 3 d after confluence, HMVEC monolayer integrity was assessed in two wells by absence of Trypan blue (0.2% in 0.1% BSA) translocation from upper to lower chamber after 3 h of incubation. HMVEC were incubated overnight with conditioned medium in the lower chamber before monocytes were added in the upper chamber for 24 h. Monocytes present in the medium of the lower chamber were counted. No monocyte was present at the insert lower face.

### Phagocytosis

PKH26 (Sigma-Aldrich)-labeled mpc were seeded with autologous or heterologous MP at different mpc/MP ratio and cultures were examined at 1–4 d using both fluorescence and phase-contrast microscopy.

### ELISA

MCP-1 (Beckman Coulter), MDC (R&D Systems), and VEGF (Cytimmune Sciences Inc.) concentrations in mpc-conditioned medium were determined by ELISA. ELISA for FKN was conducted as described previously (Foussat et al., 2001). Results were corrected according to the cell number and are expressed in pg/ml for  $1 \times 10^5$  cells.

### mpc labelings

mpc were labeled with primary antibodies for 2 h: 10  $\mu$ g/ml anti-MCP1, 50  $\mu$ g/ml anti-FKN, 10  $\mu$ g/ml anti-MDC, 10  $\mu$ g/ml anti-VEGF, revealed using FITC-conjugated secondary antibody (1:100 dilution; Jackson ImmunoResearch Laboratories) or biotin-conjugated secondary antibody (1:150 dilution; Jackson ImmunoResearch Laboratories) and FITC-streptavidin (1:50 dilution; Vector Laboratories).

Cells were labeled with annexin-V–biotin (BD Biosciences) revealed by streptavidin-FITC (Jackson ImmunoResearch Laboratories), and further labeled with anti-CD56 antibody revealed using a goat anti-mouse TRITC antibody (1:100 dilution; Jackson ImmunoResearch Laboratories). At least 100 cells from randomly chosen fields (40 $\times$  objective) were evaluated for their labeling.

Coverslips were mounted in Vectashield containing DAPI (Vector Laboratories). Controls included incubation with whole IgGs from species of the secondary antibody (50  $\mu$ g/ml; Vector Laboratories).

### Human muscle immunohistochemistry

Cross sections of frozen adult deltoid muscle biopsy samples were labeled with mouse anti-human CD56 (1:20 dilution; NHK-1-RD1; Beckman Coulter) revealed using peroxidase Vectastain ABC kit (Vector Laboratories). The distance from the CD56+ satellite cell nucleus to the lumen center of the nearest capillary was determined on 50 satellite cells in randomly chosen fields using the KS300 Imaging software (Carl Zeiss MicroImaging, Inc.).

For double labeling, sections were labeled with mouse anti-human CD56 revealed by goat anti-mouse-FITC (1:100 dilution; Jackson ImmunoResearch Laboratories) and were further labeled for MCP1, FKN, MDC, VEGF, and uPAR, revealed using TRITC-conjugated antibodies as described in mpc labelings section.

### Cell proliferation

mpc were cultured with MP in Ham's F12 medium, or with MP-conditioned medium containing 1  $\mu$ Ci/ml [ $^3$ H]thymidine for 18 h. 50  $\mu$ l trypsin-EDTA was added, radiolabeled DNA was recovered on MultiScreen Harvest plates (Millipore) using a manual Harvester (PerkinElmer) and quantified in a  $\beta$  counter.

### Oligosomal DNA levels

mpc were cultured with MP in Ham's F12 medium, or with MP-conditioned medium for 18 h, and treated using the Cell Death kit (Roche Diagnostic).

### DNA array

Total RNA was prepared from mpc at days 7 and 14 of culture using the RNeasy mini kit (QIAGEN). All further steps were performed according to the manufacturer's instructions in the Atlas Human Hematology/Immunology array (BD Biosciences) kit. For days 7 and 14 samples, 9 and 7  $\mu$ g of total RNA gave roughly similarly labeled cDNA: 989,000 and 963,000 cpm, respectively, that were deposited on membranes. Results were read using a PhosphorImager (Amersham Biosciences) after a 4-d exposure time. Analysis was performed using Image Quant software (Amersham Biosciences), that allows background noise subtraction, correction for the variation of density for housekeeping genes (all genes showed the same intensity variation between the two membranes), and finally, comparison of densitometric signals. Results were expressed in arbitrary units.

### RT-PCR

2  $\mu$ g of total mpc RNA was reverse transcribed and amplified using OneStep RTPCR (QIAGEN) and specific primers. For FKN (primers in Lucas et al., 2001) and MDC (primers in Katou et al., 2001), amplification was performed at 94, 64, and 72°C for 30 s, 30 s, and 1 min, respectively, for 38 cycles. For MCP-1 (GenBank/EMBL/DDJB accession no. M24545), the sense primer used was 5'-CCAGTCACCTGCTGTTAT-3' and the anti-sense primer was 5'-AATTCCTCAAGTCTCTGTATCTA-3', amplification was performed at 94, 55, and 72°C for 30 s for 38 cycles. For VEGF (primers in Bausero et al., 2000), amplification was performed at 94, 60, and 72°C for 30 s, 30 s, and 45 s, respectively, for 45 cycles. 10  $\mu$ l of amplification products were subjected to electrophoresis on 2% agarose and stained with ethidium bromide for visualization.

### Statistical analysis

Excepted DNA array, all experiments were performed using at least three different cultures. The *t* test was used for statistical analyses. *P* < 0.05 was considered significant.

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