Targeting Tumor-Associated Macrophages and Inhibition of MCP-1 Reduce Angiogenesis and Tumor Growth in a Human Melanoma Xenograft

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Chemokines such as monocyte chemoattractant protein (MCP)-1 are key agonists that attract macrophages to tumors. In melanoma, it has been previously shown that variable levels of MCP-1/CCL2 appear to correlate with infiltrating macrophages and tumor fate, with low to intermediate levels of the chemokine contributing to melanoma development. To work under such conditions, a poorly tumorigenic human melanoma cell line was transfected with an expression vector encoding MCP-1. We found that M2 macrophages are associated to MCP-1^{+} tumors, triggering a profuse vascular network. To target the protumoral macrophages recruitment and reverting tumor growth promotion, clodronate-laden liposomes (Clod-Lip) or bindarit were administered to melanoma-bearing mice. Macrophage depletion after Clod-Lip treatment induced development of smaller tumors than in untreated mice. Immunohistochemical analysis with an anti-CD31 antibody revealed scarce vascular structures mainly characterized by narrow vascular lights. Pharmacological inhibition of MCP-1 with bindarit also reduced tumor growth and macrophage recruitment, rendering necrotic tumor masses. We suggest that bindarit or Clod-Lip abrogates protumoral-associated macrophages in human melanoma xenografts and could be considered as complementary approaches to antiangiogenic therapy.

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INTRODUCTION

Monocyte chemoattractant protein-1 (MCP-1/CCL2) plays a critical role in the recruitment and activation of macrophages during acute inflammation (Mantovani *et al.*, 2004). It has also been shown that, in melanoma cell lines transduced with MCP-1 gene that secrete different levels of the chemokine, MCP-1 action is biphasic. Whereas high levels promote tumor rejection, low or intermediate levels support tumor growth (Nesbit *et al.*, 2001). Macrophages are important components of the innate immunity against tumors, which are attracted by locally secreted chemokines (Brigati *et al.*, 2002; Coussens and Werb, 2002). Owing to their plasticity, the macrophage functional program appears to be defined by the presence of growth factors in the tumor microenvironment as well as by intercellular interactions (Mills *et al.*, 2000). Consequently, there is a duality in the functional value of tumor-associated macrophages. When totally polarized to M1, macrophages are involved in type I reactions and may kill tumor cells. On the other hand, M2 macrophages tune up inflammation, produce angiogenic factors and metalloproteases, induce a certain degree of immunosuppression, and stimulate tumor growth (Mills, 2001; Brigati *et al.*, 2002; Mantovani *et al.*, 2002).

Human melanoma is a disease with extremely variable growth rates *in vivo* and on the basis of the above-mentioned results it seemed worth investigating (a) the type of macrophages that arrive to the tumor, (b) how to modulate macrophage infiltration, and (c) how the expression level of MCP-1 could be inhibited to diminish tumor growth rate.

To approach these issues, we have used the human melanoma cell line IIB-MEL-J as a model (Gazzaniga *et al.*, 2001), which has no production of MCP-1 (Gazzaniga *et al.*, unpublished results) and which grows slowly when xenografted into nude mice. We have transfected this cell line with an MCP-1-expression vector and obtained an intermediate MCP-1 producer, which grows faster *in vivo*. Bindarit (2-methyl-2-((1-(phenylmethyl)-1*H*-indazol-3yl) methoxy) propanoic acid) inhibits MCP-1 production *in vitro* and *in vivo* (Guglielmotti *et al.*, 2002) and the anti-MCP-1 action results in beneficial effects in models of some diseases (Guglielmotti *et al.*, 2002; Bathia *et al.*, 2005). This suggests that the blockage of this chemokine production or its

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Abbreviations: Clod-Lip, clodronate-laden liposomes; EC, endothelial cell; MCP, monocyte chemoattractant protein; iNOS, inducible nitric oxide

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action is a major target for pharmacological intervention in different human diseases, raising the possibility of therapeutic strategies to target specific macrophages attracted by the chemokine MCP-1 at the tumor site. Clodronate (dichloromethylene–biphosphonate) encapsulated into liposomes can be delivered so that it is captured by phagocytic cells. Killing of these cells is the result of intracellular accumulation and irreversible metabolic damage that causes apoptotic death (van Rooijen *et al.*, 1996). Here, we provide direct evidence that M2 macrophages are present locally in MCP-1-producing melanoma, promoting vascularization and tumor growth. Most importantly, targeting macrophages or MCP-1 production through two different pharmacological approaches renders tumors less aggressive with a clear reduction of intratumoral vascular network.

RESULTS

Transfection of the human IIB-MEL-J melanoma cell line

To establish a stable model to evaluate the contribution of MCP-1 to tumor growth, we have chosen the melanoma cell line IIB-MEL-J, which does not produce the chemokine. The level of MCP-1 released to the cell culture supernatant was less than 12 pg/ml/10^6 cells/48 hours. After lipofection with the pLXSN-MCP-1 plasmid, we were able to select a stable subline, IIB-MEL-J-MCP-1, which expressed MCP-1 mRNA and released 940 pg/ml/10^6 cells/48 hours. Mock-transfected cells (with the empty vector pLXSN) retained the characteristics of the parental line in terms of MCP-1 production (Figure 1a). Every experiment conducted thereafter, included parental and mock-transfected IIB-MEL-J as independent groups and will be referred as control lines along the work.

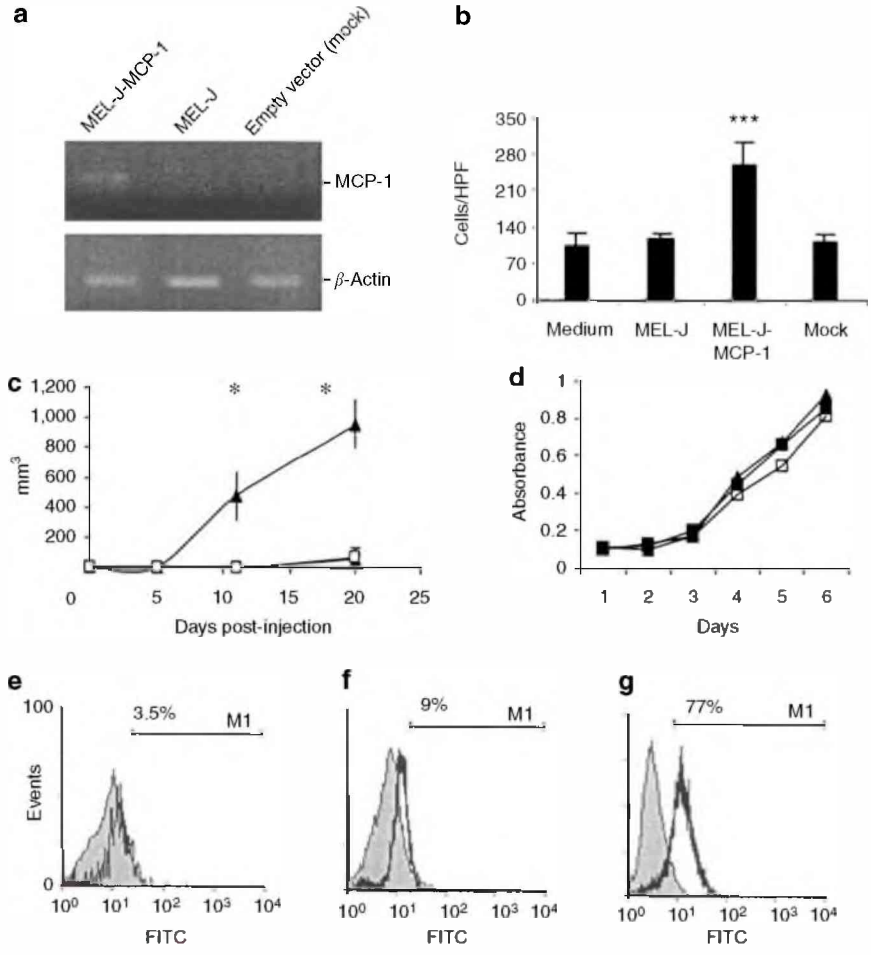

Figure 1. Characterization of IIB-MEL-MCP-1-transfected cell line. (**a**) Expression of MCP-1 mRNA by melanoma cell lines. The amplified specific fragment of 235 bp is present in the permanently transfected IIB-MEL-J-MCP-1. Analysis of β-actin served as internal control. (**b**) Chemoattractant capacity of serum-free conditioned media from the different melanoma lines assayed in a chemotaxis chamber. The number of migrated macrophages after 2 hours was counted in five high-power fields. Determinations were done by triplicate (***$P<0.0001$, Student's *t*-test). (**c**) Tumor dimensions were measured with a caliper and for microscopic tumor masses, dimensions were taken on hematoxylin–eosin sections with reticulated ocular in 5–10 different tumor samples. Triangle: IIB-MEL-J-MCP-1, square: IIB-MEL-J and mock-transfected lines. (*$P=0.015$, Wilcoxon's rank-sum test). (**d**) *In vitro* growth rate: 50,000 cells of each line were plated onto 96-well dishes and cultured in complete melanoma medium. Cells were quantified at different time points with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Full square: parental line; full triangle: IIB-MEL-J-MCP-1; empty square: mock-transfected line. Determinations were done by quadruplicates. CCR2 expression in (**e**) control, (**f**) IIB-MEL-J-MCP-1, and (**g**) 1G11 lines. Gray histogram corresponds to isotype control and white histogram to the reactivity to CCR2 chemokine receptor with rabbit mAb. Percentages depicted above the marker line indicate the fraction of CCR2-positive cells.

To test whether the chemokine released by IIB-MEL-J-MCP-1 was functional, serum-free culture supernatants were seeded in the lower compartment of a chemotaxis chamber. Monocyte/macrophage migration was only induced by IIB-MEL-J-MCP-1-conditioned medium (Figure 1b) and blocked when supernatants were preincubated with a specific antibody against MCP-1 (data not shown). Control lines-conditioned media were not chemoattractant for monocytes/macrophages (Figure 1b). When injected subcutaneously into nude mice, IIB-MEL-J-MCP-1 developed larger tumors than those of control cell lines: tumors attained statistical significant differences ($P=0.015$) after 11 days of inoculation up to the end of the observation period (day 20) (Figure 1c). This increased growth velocity could not be attributed to an alteration of cell properties after transfection. *In vitro* growth kinetics of IIB-MEL-J-MCP-1 and control cell lines were similar (Figure 1d). In addition, the main receptor for MCP-1, CCR2, was absent (Figure 1e and f) discarding a putative autocrine role in tumor growth rate.

Inflammatory infiltration and angiogenesis

Tumor samples were obtained at different intervals to examine the occurrence and localization of inflammatory infiltrate. Recruitment of macrophage-like cells within and around tumors was significantly higher ($P=0.01$) in MCP-1-transfected tumors than in control tumors from early stages of tumor establishment until the end of the observation period (Figure 2a and b and Table 1). Eleven days after IIB-MEL-J-MCP-1 injection, the macrophage-like cells recruitment persisted and was accompanied by fibroblasts and edema, alterations that were absent in control tumors (data not shown). There was scarce presence of peritumoral lymphocyte-like cells, although polymorphonuclear neutrophils increased peritumorally 4 days post-IIB-MEL-J-MCP-1 cells implantation (Table 1). The above-described inflammatory infiltrate was absent in control tumors.

To analyze the phenotypic identity of the mononuclear infiltrates and the degree of vascularization, consecutive tumor sections were stained with primary antibodies to detect CD31, F4/80, and CX_3CR1 expression. Four days after IIB-MEL-J-MCP-1 injection, a peripheral border of incipient vessels appeared as well as isolated CD31 + cell clusters (Figure 2c). Concerning F4/80, a striking positivity was found around tumor mass and also among neoplastic cells (Figure 2e). For CX_3CR1, a fainter staining was present peri- and intratumorally partially overlapping with F4/80 areas (data not shown).

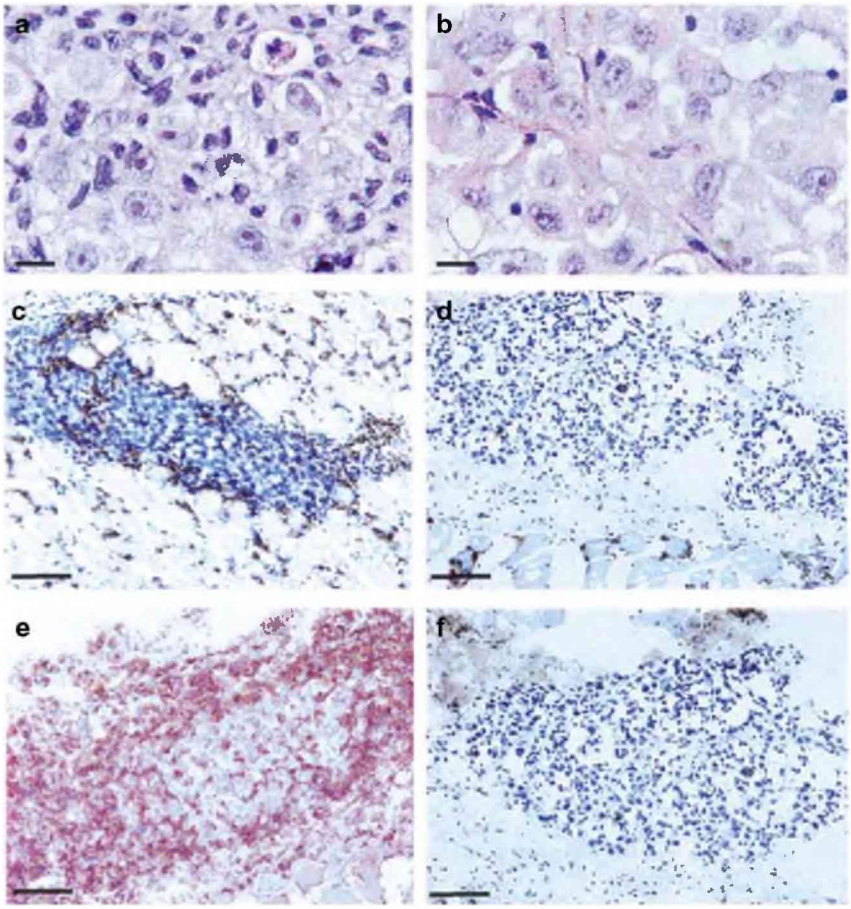

Figure 2. Inflammatory infiltration and angiogenesis. (**a**) Mononuclear cells are mainly present among IIB-MEL-J-MCP-1 cells (11 days growing tumors) (**b**) Control tumors present no mononuclear infiltration. Bar = 10 μm. Immunohistochemical analysis identifying (**c** and **d**) CD31 + and (**e** and **f**) F4/80 + cells in 11-day (**c** and **e**) IIB-MEL-J-MCP-1 (**d** and **f**) and control melanoma. Bar = 125 μm.

Table 1. Quantitative analysis of peritumoral and intratumoral infiltrating cells[1]

	Peritumoral					
	Macrophage-like cells		Lymphocyte-like cells		Polymorphonuclear cells	
Days	Control	MCP-1+	Control	MCP-1+	Control	MCP-1+
4	2 (0–11)	4** (0–26)	0 (0–4)	0 (0–3)	0 (0–3)	2** (0–22)
11	1 (0–4)	4** (0–14)	0	0	0 (0–2)	0 (0–2)
20	2 (0–20)	33** (0–70)	0 (0–2)	0 (0–2)	0 (0–3)	0 (0–6)
			Intratumoral			
4	2 (0–10)	5** (0–33)	0 (0–2)	0 (0–2)	0 (0–3)	0 (0–8)
11	2 (0–6)	3* (0–9)	0	0	0 (0–1)	0
20	0 (0–2)	8** (0–30)	0 (0–1)	0 (0–1)	0 (0–1)	0

MCP, monocyte chemoattractant protein.
[1]Counts were performed in 10 fields of original magnification × 1000. Values correspond to medians and ranks. *P=0.05 and **P=0.01 (Wilcoxon's rank test).

Table 2. Immunoreactive cells in 11-day tumors[1]

		Vessels	CD31+ clusters	F4/80+ cells	CX3CR1+ cells
IIB-MEL-J-MCP-1	Intratumorally	+++	+++	140	35
	Peritumorally	+++	+++	242	37
Control	Intratumorally	–	–	2	0
	Peritumorally	+++	+++	174	42

[1]Total cell counts performed at 10 high-power fields and vascularization at original magnification × 200 fields. Vascular structures and cell clusters were ranked as: + (<10 elements); ++ (10–20 elements), and +++ (>20 elements).

When 11-day IIB-MEL-J-MCP-1 tumors were analyzed, a strong positivity for CD31 was detected and revealed a massive branched intratumoral vascular network. This important vascularization was completely absent in control tumors where MCP-1 was not produced. Surprisingly, no differences were found peritumorally in terms of CD31+ vessels and cell clusters (Table 2). The intratumoral differences between IIB-MEL-J-MCP-1 and control tumors were also extensive to F4/80 and CX_3CR1 macrophages infiltration. Surrounding macrophages were present in control tumors, although recruitment was increased in those generated from cells transfected for MCP-1 expression (Table 2). Eleven days post-injection of neoplastic cells, tumors showed a well-established angiogenic network. Consequently, we focused on days 4 and 11 as a prevascularized and vascularized time points in tumor development, respectively.

The immunohistochemical analysis revealed the presence of cells identified as macrophages but this marker positivity does not reveal their functional significance in tumor tissues. For this purpose, two characteristic enzymes were determined. Type I macrophages (M1 macrophages), which synthesize inducible nitric oxide (iNOS) but not arginase, were proposed to be tumoricidal macrophages, whereas M2 macrophages, which synthesize arginase but not iNOS, would be protumoral (Sironi *et al.*, 1999; Schmeisser *et al.*, 2001; Koide *et al.*, 2004).

To determine which type of infiltrating macrophages are present at IIB-MEL-J-MCP-1 melanomas, extracts from 4-day tumor sites were prepared to test *in vitro* arginase and iNOS activities (Figure 3). Macrophages infiltrating IIB-MEL-J-MCP-1 tumors presented a significant reduction of nitric oxide (P=0.005) and arginase tended to be augmented with respect to control tumor sites, although this difference does not reach statistical signification (Figure 3).

Depletion of macrophages with Clod-Lip

To determine how critical macrophages were to the increased growth ability of MCP-1-transfected melanoma, mice were depleted of phagocytic cells by the administration of Clod-Lip as described. Intravenous and local injections of Clod-Lip were intended to eliminate early recruitment of macrophages, which could be attracted by the chemokine released once transfected tumor cells were injected. Under this condition, IIB-MEL-J-MCP-1 tumors grown in mice treated with Clod-Lip showed a reduction superior to 70% (P=0.0003, Student's t-test) in tumor bulk (Figure 4a) as compared with tumor growth in undepleted mice. Mice survival also increased with macrophage depletion (data not shown). Growth rates for parental or mock-transfected tumors remained unchanged with treatment (Figure 4b).

To verify the intensity of macrophage depletion obtained by Clod-Lip, tumor sites from Clod-Lip-treated and untreated

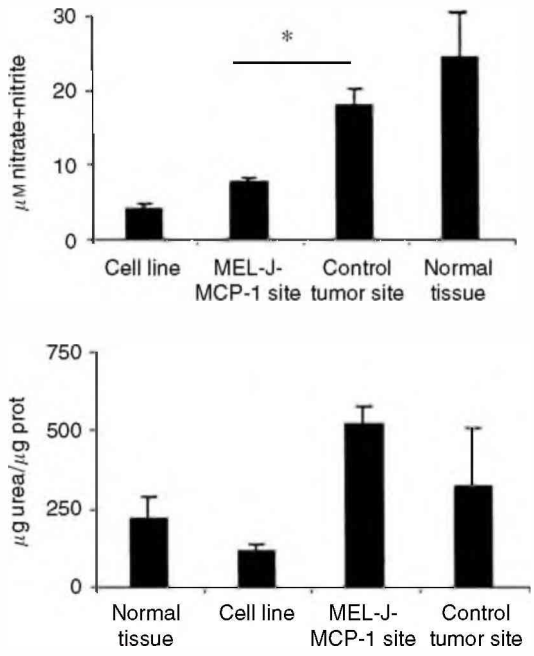

Figure 3. Infiltrating macrophages profile. Four-day tumor sites or normal tissue were processed and analyzed for arginase content as a measure of urea production and nitric oxide. Results were expressed as mean±SD. (*$P=0.005$, Student's *t*-test).

mice could not be stained with F4/80 owing to intense necrosis and chromogen deposition. Instead, a fluorochrome-labelled CD11b mAb was used. Abundant positive macrophages were easily identified at the tumor site in 4-day IIB-MEL-J-MCP-1 but were scarcely present in Clod-Lip-treated tumor (Figure 4c). To investigate whether macrophage depletion had an impact on vascularization, tumor biopsies were analyzed for CD31 expression (Figure 4d). A deep reduction in angiogenesis was observed with respect to nontreated tumors. Tumor vessel circumferences were significantly reduced (64.57 ± 5.86 *vs* 128.12 ± 9.13 μm; $P=0.003$) (Figure 4d inset and 4e). In contrast, control tumors did not present variations of vessels morphology with treatment (not shown). To discard a direct cytostatic/cytotoxic effect of Clod-Lip on melanoma cells or endothelium, cells were incubated *in vitro* with Clod-Lip or phosphate-buffered-saline-Lip. No significant effect on viability was recorded neither on different tumor nor endothelial cells (ECs) by Clod-Lip exposure during 30 (Figure 4f) or 72 hours (not shown), whereas this treatment was cytotoxic for macrophages and polymorphonuclear cells ($P=0.05$, Figure 4f).

Bindarit treatment

Even when an important reduction in tumor burden was obtained with Clod-Lip as a consequence of disrupting macrophages recruitment to tumor site, there was an incipient angiogenesis characterized by smaller vessels. This precarious microvascular development could be because of some angiogenic potency of MCP-1 itself. To address this issue, mice were injected intraperitoneally with bindarit at a dose of 100 mg/kg twice a day, beginning the same day that tumor cells were subcutaneously inoculated. Bindarit treatment was maintained until the animals were killed to sample tumor tissues for histology. The analysis revealed a reduction in IIB-MEL-J-MCP-1 tumor volume with respect to control tumors, both at days 4 and 11 post-treatment ($P=0.05$, Wilcoxon's rank test) (Figure 5a). Exclusively, tumors expressing MCP-1 were almost completely necrotic when examined at days 4 and 11 after treatment ($P=0.0022$ and 0.01) (Figure 5b and c). The combination of bindarit and Clod-Lip did not improve this result (data not shown). Macrophages recruitment was abolished by bindarit (Figure 5c). Control lines were not affected by the drug (data not shown). Bindarit, even at the maximal concentration, did not exhibit a direct *in vitro* cytotoxic effect on melanoma (Figure 6a and b) or ECs (Figure 6c), although it inhibited MCP-1 expression (Figure 6d).

Effect of MCP-1 on ECs

The results obtained above suggest that MCP-1 plays a role to vascularize IIB-MEL-J-MCP-1 tumors, as there is still a precarious neovessel development even after reducing macrophages with Clod-Lip. To assess whether MCP-1 could induce vascularization, murine microvascular ECs were incubated with different concentrations of recombinant MCP-1. No induction of proliferation was found (Figure 7a), discarding its contribution as a mitogenic agent on ECs. However, when the same microvascular ECs were incubated in matrigel, tubular structures were induced to organize in the presence of recombinant chemokine (100 ng/ml) (Figure 7b) or conditioned supernatants from IIB-MEL-J-MCP-1 (data not shown). A specific antibody against MCP-1 aborted ECs organization in tubules and only precarious buds were observed emerging from cell clusters (Figure 7c). Tubule structures were not altered by the addition of an isotype control antibody and no structures were formed when microvascular ECs were grown alone or after adding conditioned supernatants from control lines (not shown).

DISCUSSION

MCP-1 released by cancer cells recruits macrophages into tumor tissue (Desbaillets *et al.*, 1994; Negus *et al.*, 1997; Valkovic *et al.*, 1998). The role of this chemokine in tumor progression and metastasis is a subject of interest in prognosis as well as in the development of possible antitumor strategies (Varney *et al*, 2002; Ohta *et al.*, 2003; Koide *et al.*, 2004; Colombo and Mantovani, 2005; Kuroda *et al.*, 2005).

Macrophages respond to the released chemotactic environmental signals with diverse functional programs (Mills *et al.*, 2000) and this inflammatory scenario has been associated with neoplasia (Coussens and Werb, 2002; Balkwill *et al.*, 2005).

In this study, we performed transfection of the human melanoma cell line IIB-MEL-J (negative for MCP-1 production) with the purpose to have a MCP-1 intermediate-producer melanoma model to distinctively analyze the contribution of this chemokine in the profile of recruited macrophages and the effect on tumor growth. The comparison of different melanoma cell lines with spontaneous

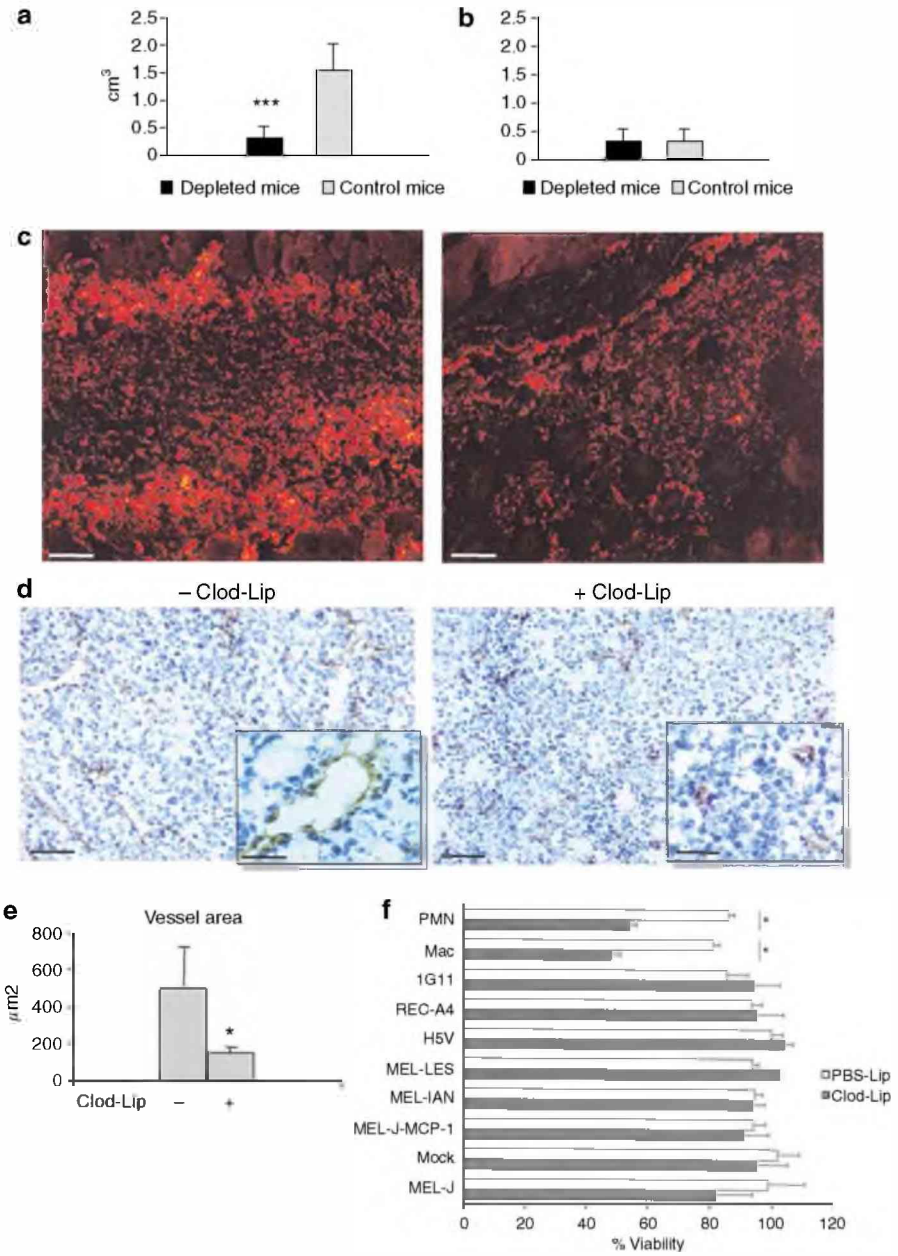

Figure 4. Clod-Lip treatment. Tumor dimensions after macrophage depletion with Clod-Lip in (**a**) IIB-MEL-J-MCP-1 and (**b**) control tumors. The day before tumor cell injection, mice were injected with 200 μl of clodronate liposomes intravenously and 50 μl subcutaneously in the region where tumor cells would be injected. Depletion was maintained by systemic administration of 200 μl Clod-Lip dose once per week. **(c)** Macrophage recruitment in 4-day IIB-MEL-J-MCP-1 is highly diminished after Clod-Lip treatment. Cryostat tissue sections were incubated with CD11b-TRITC. Bar = 100 μm. (**d**) Tumor angiogenesis in Clod-Lip mice. Tumor sections from IIB-MEL-J-MCP-1 tumor grown in Clod-Lip-treated and untreated mice were immunostained for CD31 detection. (Bar = 100 μm, insets bars: 100 μm). (**e**) Quantification of tumor vessels areas was performed on immunostained sections with Kontron Electronics software coupled to an image analyzer. (**f**) *In vitro* Clod-Lip cytotoxicity: cells were incubated in the presence of Clod-Lip (gray bars) and phosphate buffered-saline–Lip (white bars) in 5% complete medium and exposed for 29 hours at 1.5 μl of Clod-Lip (9 μg) or phosphate buffered-saline–Lip. Cell viability was assayed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and absorbances read at 595 nm. Values correspond to the mean of quadruplicates. Two independent experiments were performed. (*$P < 0.04$, Student's *t*-test).

capacity to produce MCP-1 could be confusing as there are several angiogenic and antiangiogenic factors that could not be kept as constants. Most importantly, in this work, we focused on the effect of Clod-Lip and bindarit as putative agents to abolish protumoral effect of macrophages.

At the light of our results, the significant presence of intratumoral macrophages during the early stages of tumor progression and their persistence during evolution seem to be responsible for tumor growth promotion. We found that tumor dimensions of IIB-MEL-J-MCP-1 tumors enhanced 3.5-

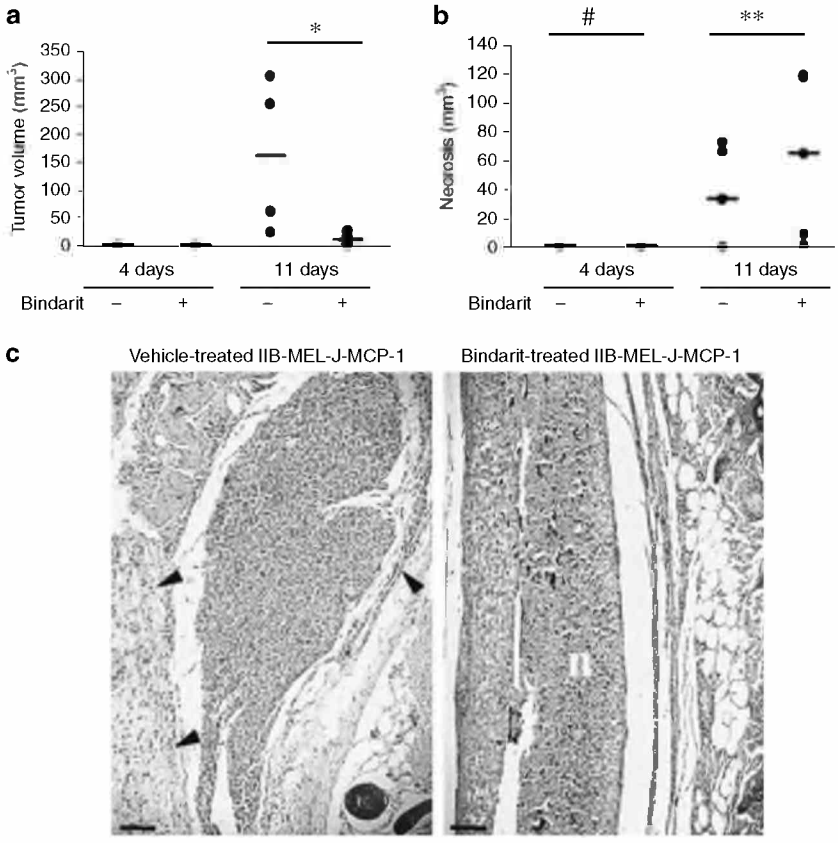

Figure 5. Bindarit treatment. Analysis of (**a**) tumor volume and (**b**) necrotic regions in IIB-MEL-J-MCP-1 tumor-bearing mice. Biopsies were obtained at 4 and 11 days after treatment, counterstained with hematoxylin–eosin, and dimensions measured with microscope caliper. $^{*}P=0.05$, $^{\#}P=0.01$, $^{**}P=0.0022$. (**c**) Representative images of 11-day IIB-MEL-MCP-1 tumors. A tumor from vehicle-treated animals appeared surrounded by macrophage infiltration (arrowheads) and a tumor from bindarit-treated mice depicted profuse necrosis (*n*) and no macrophage infiltration at the periphery. Bar = 200 μm.

and 18-fold after 4 and 11 days, respectively. The absence of CCR2 expression in melanoma cells discards the possibility of autocrine effect of the chemokine released by IIB-MEL-J-MCP-1 cells.

Apart from F4/80-positive cells recruitment, an important afflux of CD31+ cells was present nearby the incipient tumors. We also found a strong reduction in the levels of nitric oxide in IIB-MEL-J-MCP-1 tumors and an augmented arginase activity, outlining the presence of M2 macrophages recruited to the tumor. We propose that IIB-MEL-J-MCP-1 tumors grew highly vascularized owing to the presence of M2 macrophages, which could be contributing with an important source of angiogenic factors (Schmeisser *et al.*, 2001; Coussens and Werb, 2002; Varney *et al.*, 2005) or favoring vasculogenesis (Anghelina *et al.*, 2004; Anghelina *et al.*, 2006).

The protumoral effect of infiltrating macrophages in IIB-MEL-J-MCP-1 was confirmed by Clod-Lip treatment results. We observed a substantial reduction in tumor growth MCP-1-transfected melanoma xenografts after macrophage depletion. We did not find direct cytotoxicity on melanoma cells or endothelial employing Clod-Lip, which implies that the drug is captured selectively by phagocytic cell and there is no uptake by the other two cell types in the tumor. Clodronate is a non-nitrogen biphosphonate and when encapsulated, it is metabolized intracellularly to a β–γ methylene analog of adenosine triphosphate, that is cytotoxic to macrophages *in vitro* (van Rooijen *et al.*, 1996; Lehenkari *et al.*, 2002). Without encapsulation, clodronate exerts no inhibition in cell proliferation either *in vitro* or *in vivo* in solid tumors (cutaneous and uveal melanomas) (Knight *et al.*, 2005).

Instead, nitrogen-containing biphosphonates had a direct effect through induction of apoptosis on tumor cells (Yamagishi *et al.*, 2004; Knight *et al.*, 2005).

Thus, the choice of Clod-Lip allowed dividing clearly the role of macrophages respect to a direct influence on tumor cells.

Even when there was a significant tumor reduction in IIB-MEL-J-MCP-1 with Clod-Lip treatment, we found that there was still perfusion through a poor vessel network with very small calipers. As it has been previously shown, the origin of this modest vascular formation could be owing to EC migration and/or sprouting from local residing ECs or recruitment of circulating endothelial progenitors (Asahara *et al.*, 1997; Skovseth *et al.*, 2002; Spring *et al.*, 2005).

By using bindarit, a molecule that has been shown to inhibit MCP-1 production both *in vitro* and *in vivo* (Sironi *et al.*, 1999; Guglielmotti *et al.*, 2002; Bathia *et al.*, 2005), we

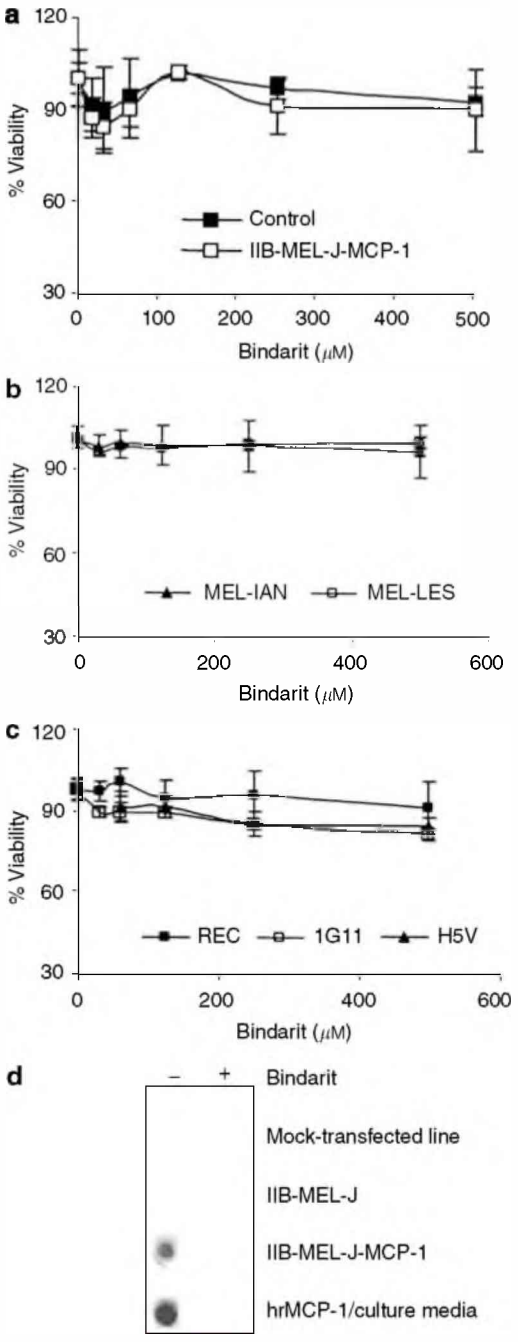

Figure 6. Evaluation of bindarit cytotoxicity *in vitro*. Viability of (**a**) IIB-MEL-J-MCP-1 and control lines; (**b**) IIB-MEL-IAN and IIB-MEL-LES, and (**c**) EC lines were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Values correspond to the mean of quadruplicates. Two independent experiments were performed. (**d**) Immunodot detection of MCP-1 production in 48 hours-conditioned supernatants of the different melanoma cell lines cultured in absence or presence of bindarit (500 μM). Human recombinant MCP-1 (200 ng, Peprotech) was seeded as positive control and culture media as negative control.

could abolish such vascularization, rendering tumors almost completely necrotic. When necrosis was not complete, the scarce viable remaining tumor cells were exclusively located at the periphery of the tumor mass. As no direct cytotoxicity appeared from *in vitro* assays, the effect of bindarit treatment was assumed to happen because of the lack of attracting signals for the macrophages.

It was observed, in a macrovascular endothelium model as human umbilical vein endothelial cells (Salcedo *et al.*, 2000) and in this paper using microvascular ECs (Figure 7), that MCP-1 induces ECs to arrange spatially forming tube-like structures without evident proliferation (Figure 7). This behavior is MCP-1-specific provided that the tubularization process was blocked by a specific antibody to the chemokine but not by the appropriate isotype control. Consequently, according to our results, MCP-1 would be predisposing ECs to an "angiogenic state" but the signals to proliferate would come with the presence other angiogenic component/s of tumor microenvironment. Vascular Endothelial Growth Factor (VEGF) could be one of these candidates, as it is expressed by IIB-MEL-J melanoma cells and transfectants (data not shown). The monocyte is an example of a non-EC type that is positive for VEGF R1, active on monocyte chemotaxis (Mantovani *et al.*, 2004). According to these evidences, it is tempting to speculate that, when only VEGF is present, melanoma vascularizes poorly, as it occurs with IIB-MEL-J tumors. Contrarily, with MCP-1 adjuvancy, more macrophages reach the tumor site to trigger a profuse vascular network, permitting a more aggressive tumor development.

This role of MCP-1 in orchestrating recruitment of M2 macrophages but, above all, tumor vascularization by multiple ways drives the attention to future therapies using drugs like Clod-Lip or bindarit, which could block or reduce the inflammatory effects of this chemokine.

MATERIALS AND METHODS

Melanoma cell lines

The human melanoma cell lines IIB-MEL-J and transfectants, IIB-MEL-IAN and IIB-MEL-LES were maintained in DMEM: Ham F12 medium (1:1), supplemented with 10% fetal bovine serum (FBS), 20 nM sodium selenite, 100 μM ascorbic acid, 0.15 mg/ml sodium pyruvate, 0.3 mg/ml galactose, 5 μg/ml insulin, and 2 mM glutamine (melanoma medium) as previously reported (Gazzaniga *et al.*, 2001). Cells were regularly tested to be mycoplasma-free. Transfection was performed with Lipofectamine™ (Life Technologies, Gaithersburg, MD) with the pLXSN vector bearing a 700-bp long fragment of human MCP-1 cDNA or with the empty vector (mock transfection). Cultures were then selected with 800 μg/ml G418 (Life Technologies), cloned, and expanded as cell lines. For tumor generation, 1.5×10^6 cells were subcutaneously injected in athymic male NIH (S)-nu mice (6–8 weeks old, obtained from the Faculty of Veterinary Sciences Facility, University of La Plata, Buenos Aires). Animal handling and care were conducted in conformity with national and international policies.

MCP-1 and CCR2 determinations

Released MCP-1 was quantified with commercially available antibodies for ELISA (BD Biosciences, NJ). Development was performed by adding chromogen substrate ABTS (KirKegaard and Perry, Gaithersburg, MD) and the plates were read at 405 nm in an automatic ELISA reader (BIORAD Laboratories Inc, Hercules, CA).

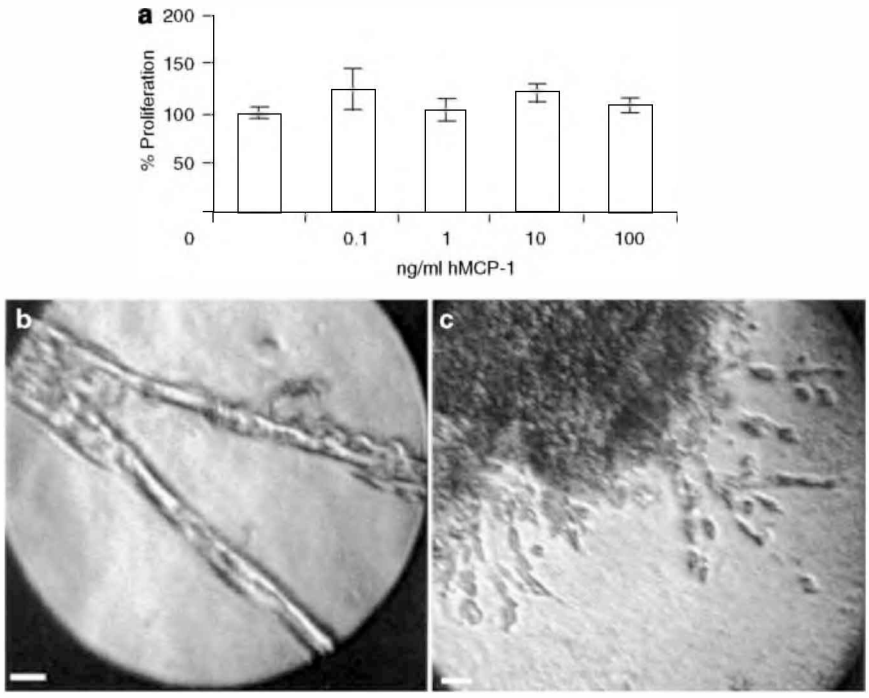

Figure 7. Effect of MCP-1 on ECs. **(a)** Microvascular proliferation in response to variable concentrations of human recombinant MCP-1. Cells were grown on gelatin-coated wells in the presence of 0.5% FBS and after 24 hours cell number was revealed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. **(b)** Microvascular ECs depicting tube formation *in vitro* after cultured for 4 days in the presence of matrigel plus 100 ng/ml of human MCP-1. **(c)** ECs aborted tubularization when grown in matrigel plus 100 ng/ml of human MCP-1 plus MCP-1 antibody (1 μg/ml). Bar = 10 μm.

MCP-1 mRNA was determined by reverse transcriptase–PCR as described by Luciani *et al.* (1998) using extracted RNA from every cell line as template and the following specific primers (forward 5′-CTCAGCCAGATGCAATCAATG-3′ and reverse 5′-GTTCAAGTCTTC GGACTTTGG-3′).

CCR2 determination was performed by FACS analysis on 1% paraformaldehyde-fixed cells using rabbit anti-human CCR2 (Abcam, Cambridge Science Park, Cambridge, UK) (1/25) and goat anti-rabbit conjugated to FITC (Sigma Chemicals Co., St Louis, MO) (1/140). Results were analyzed with WinMDi program. Markers were set with an inclusion criteria of less than 5% of the isotype control population. The percentage of positive cells were calculated with the statistical function of WinMDi program.

Treatments

For macrophage depletion, a suspension of clodronate liposomes containing about 6 mg clodronate per 1 ml suspension was employed. Clodronate was a gift of Roche Diagnostics (GmbH, Mannheim, Germany) and it was encapsulated in liposomes as described earlier (van Rooijen and Sanders, 1994). Other reagents for preparation of liposomes are: phosphatidylcholine (Lipoid, GmBH, Germany) and cholesterol (Sigma Chemicals Co.,). Clodronate liposomes (200 and 50 μl) were administered intravenously (by retroorbital puncture) and subcutaneously (to deplete local draining areas) the day before tumor cells injection and thereafter every 5–7 days. For *in vitro* assays, 9 μg/well were employed.

Bindarit (Angelini Farmaceutici, ACRAF SpA, Italy) was intraperitoneally administered twice daily at a dose of 100 mg/kg weight (12.5 ml/kg body weight). The control group received vehicle (0.5% methylcellulose). Mice were killed at day 4 or 11 and tumors were fixed in 10% buffered formalin. Tumor sizes and necrosis were measured with a microscope caliper. Experiments (either with Clod-Lip treatment or bindarit) were repeated twice and 4–6 animals were included in each group. For *in vitro* assays, the compound was resuspended in melanoma culture medium supplemented with 5% FBS, added to cell cultures at 70–80% confluency, and incubated for 48 hours. Cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and culture supernatants were evaluated for MCP-1 production.

Tissue processing

For every group, tumors were bisected: one half of each specimen was fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4–7 μm, and stained with hematoxylin–eosin. The other half was frozen and stored at −80°C for immunohistochemistry. Briefly, acetone-fixed tumor sections were blocked with 5% normal rabbit serum and incubated with optimal dilutions of the primary antibodies. Rat anti-mouse mAbs used were MEC 13.3 (BD Biosciences, Pharmingen, San Diego, CA) for CD31 detection, F4/80 (Serotec Ltd., Oxford UK) for macrophages and anti-CX_3CR1 fractalkine receptor (BD Biosciences). The specific binding was revealed using 1/100 goat anti-rat IgG (Sigma Chemicals Co.), 1/500 rat peroxidase–antiperoxidase complex (Accurate Chemicals, Westbury, NY), and 0.05% 3,3′-diaminobenzidine (Sigma Chemicals Co.) plus 0.03% H_2O_2. Endogenous peroxidase activity was blocked with 0.3% H_2O_2. All reagents except primary antibodies were used as controls to confirm specific antibody binding.

Quantitative evaluations

Tumor bulk *in vivo* was reported as volume, determined by measurement of the smallest and largest dimensions of the tumors with a caliper. For microscopic tumors, dimensions were measured with a reticulated ocular on counterstained tissue samples by hematoxylin and eosin. Volumes were calculated as $(a \times b^2)/2$, where a is the larger dimension and b is the smaller dimension. Determination of vessels areas were performed with an image analyzer (KS 300 Kontron Electronik) coupled to an Axioplan microscope (Zeiss, Germany). Cells were counted in at least 10 fields/sample at original magnification $\times 1000$.

NO and arginase assay

NO released in the cultures was measured by Griess reaction as the amount of NO_3^- and NO_2^- produced by using a nitrate/nitrite assay kit (Cayman Chemical, Ann Arbor, MI). Arginase activity was measured in cell lysates as described by Corraliza *et al.* (1994).

EC viability

Ten thousand murine 1G11 ECs (Dong *et al.*, 1997) and REC-A4 ECs (Gazzaniga *et al.*, 2004) were plated on gelatin-coated wells, left overnight for adherence in DMEM medium supplemented with 0.5% FBS, 2 mM glutamine, 1% nonessential amino acids and 2 mM sodium pyruvate. H5V (Garlanda *et al.*, 1994) were grown in DMEM medium supplemented with 2 mM glutamine and 0.5% FBS and plated without coating. Subsequently, cells from every cell line were incubated for 24 hours with different drugs (bindarit, Clod-Lip, or vehicles). After incubation, living cells were revealed by incubation with 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemicals Co.) and absorbances read at 595 nm. Determinations were done by sextuplicate.

Tube-like structures in matrigel

Fifty thousand ECs (1G11) were plated on coverslips together with matrigel (BD) (1.5 mg/ml) diluted in rhMCP-1 (Peprotech, Rocky Hill, NJ), melanoma cells-conditioned medium. A mAb against MCP-1 antibody (BD) (1 μg/ml) was used to block MCP-1. Plates were monitored everyday and photographs were taken at day 4 after fixation with 1% paraformaldehyde in phosphate-buffered solution.

Chemotaxis assay

Migration of cells was evaluated using 5-μm pore size filters (Neuroprobe Inc, Gaithersburg, MD) placed in a Boyden chamber. Every melanoma line was grown till 90% confluence, maintenance medium was removed and left for 48 hours in melanoma medium 1% FBS. These conditioned media were placed into the lower chamber of the Boyden chamber. Murine macrophages obtained from peritoneal cavity were resuspended in serum-free melanoma medium at a concentration of 1.5×10^6 cells/ml and 40 μl of cell suspension was placed onto the upper chamber. A mAb against MCP-1 antibody (BD) (1 μg/ml) was used to ensure specific migration. After an incubation of 2 hours at 37°C in 5% CO_2, the upper chamber was removed and the cells in the lower face of the filter stained and counted in five fields (original magnification $\times 400$). Determinations were done by triplicate.

Statistical analysis

In vitro data were analyzed by using Student's *t*-test and *in vivo* results with the nonparametric Wilcoxon's rank sum test using Statistix software version 8.0 (Analytical Software, Tallahassee, FL). $P \leqslant 0.05$ was considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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