Clin Exp Metastasis (2008) 25:225–231
DOI 10.1007/s10585-007-9136-0

RESEARCH PAPER

Tumoral and macrophage uPAR and MMP-9 contribute to the invasiveness of B16 murine melanoma cells

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Received: 12 June 2007 / Accepted: 30 November 2007 / Published online: 11 December 2007

Abstract The aim of this study was to investigate whether tumor cells as well as tumor-associated macrophages (TAMs) contribute to the generation of protease activities essential to tumor cell invasiveness, such as matrix metalloproteinase 2 and 9 (MMP-2 and MMP-9), and the urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR). We found that the enhanced invasiveness through Matrigel-coated filters of B16 murine melanoma cells stimulated with IFNγ was associated with an higher expression of uPAR and MMP-9 in these cells. Moreover, treatment with anti-MMP-9 or anti-uPAR monoclonal antibodies abrogated the increase of invasiveness in IFNγ-stimulated melanoma cells, suggesting a cooperation of uPA system and MMP-9 in cytokine-stimulated invasiveness. Invasiveness through Matrigel was also enhanced in B16 melanoma cells exposed to a medium conditioned by TAMs, represented in our experimental model by thioglycollate-elicited macrophages co-cultivated with melanoma cells. Macrophages isolated from these co-cultures were found to express higher levels of uPAR and MMP-9 compared to macrophage cultures alone, and the pro-invasive activity of the co-culture-conditioned medium was abrogated by anti-MMP-9 monoclonal antibodies, but not anti-uPAR monoclonal antibodies. Furthermore, the enhanced uPAR and MMP-9 expression in macrophages co-cultivated with tumor cells seems a rather specific phenomenon, generated through a cell-to-cell contact mechanism. On the whole, our data point to a cooperation between tumor cells and macrophages elicited by tumor cells themselves in generating key enzymes essential in the promotion of tumor invasiveness, such as uPAR and MMP-9.

Keywords Murine melanoma cells · Macrophages · Cell invasiveness · Matrix metalloproteinase 2 (MMP-2) and 9 (MMP-9) · Urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR)

Introduction

It is well established that the transition from a non-invasive to an invasive phenotype in tumor cells is associated with several molecular changes, including an enhanced expression of different families of proteases, e.g. MMP-2 and MMP-9, and uPA system (uPA/uPAR) [1–5].

Both families of proteases are markers of melanoma progression. Indeed, uPA/uPAR and MMPs are expressed in advanced stages of primary and metastatic melanoma lesions [6–8]. Moreover, antisense oligodeoxynucleotides for uPAR inhibit invasion and metastatic diffusion in a human melanoma line [9], while the inhibition of MMP expression by genetic [10] and pharmacological [11] approaches blocked the invasiveness of murine melanoma cells.

Different types of host cells, e.g. infiltrating lymphocytes and macrophages, are known to be present in the tumoral

This paper was presented in part at the "11th World Congress on Advances in Oncology and 9th International Symposium on Molecular Medicine" 12–14 October 2006, Hersonissos, Crete, Greece.

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microenvironment [12, 13]. Although, a role in host defense mechanisms against malignancy has been attributed to these cells [14], increasing evidences suggest, however, that tumor-associated macrophages (TAMs) may secrete various protease activities promoting the invasiveness of tumor cells [15–18]. In particular, metastatic dissemination of a mammary cancer was reduced in colony stimulating factor-deficient mice, a condition that impairs the recruitment of macrophages into the tumoral microenvironment [19].

In the present study, we explored the possibility that MMP-2, MMP-9 and uPA system are the effectors of the enhanced invasiveness expressed by B16 murine melanoma cells stimulated with IFNγ, that we reported in a previous paper [20]. We also investigated whether the enhancement of invasive properties observed in B16 melanoma cells stimulated by activated macrophages [15] is related to a release of MMP-2, MMP-9 and uPA system by these latter. To this aim, we determined MMPs and uPA system in the medium conditioned by thioglycollate-elicited murine peritoneal macrophages co-cultivated with a low metastatic clone isolated from B16-F10 melanoma line, the F10-M3 cells.

Materials and methods

Cell lines and culture conditions

In this study, we used a low metastatic clone of B16-F10 murine melanoma line, the F10-M3 cells [21], primary cultures of murine fibroblasts isolated in our laboratory from C57Bl/6 mice and HT1080 human fibrosarcoma cells (ATCC, Rockville, MD). Cells were grown in Dulbecco's modified Eagle medium containing 4500 mg/l glucose (DMEM 4500, GIBCO) supplemented with 10% fetal calf serum (FCS) (Boerhinger Mannheim, Germany), at 37°C in a 10 % CO_2-humidified atmosphere. 5.0×10^5 cells were seeded in 100 mm Falcon dishes and propagated every 3 days by incubation with a trypsin-EDTA solution (GIBCO). Cultures were periodically monitored for mycoplasma contamination using Chen's fluorochrome test [22]. In some experiments, F10-M3 murine melanoma cells were grown for 24 h in a medium supplemented with IFNγ (25–100 U/ml) (Peprotech, England), in the presence or absence of JAK inhibitor I (500 nM) or AG490 (500 nM) (Calbiochem).

Preparation of co-cultures of macrophages with murine melanoma cells or normal fibroblasts

Cultures of macrophages were established from peritoneal exudates collected by lavage from 6–8 week-old C57Bl/6 mice that had been injected intraperitoneally with 1 ml of 3% thioglycollate broth (Sigma) 3–4 days before, as reported in a previous paper [15]. 1:1 co-cultures were prepared by seeding F10-M3 melanoma cell or normal fibroblast suspensions on macrophage monolayers, at the density of 250×10^3 cells/cm^2. Macrophage cultures and macrophage co-cultures were incubated in a DMEM medium containing 250 μg/ml BSA (DMEM/BSA), at 37°C, in a 10 % CO_2-humidified atmosphere. After 24 h, tumor cells or normal fibroblasts were removed from the macrophage co-cultures by incubation with a trypsin-EDTA solution. By using anti-S-100 mAb, we found that less than 0.1% of cells contained in macrophage preparations was represented by tumor cells.

Total RNA extraction, cDNA synthesis and PCR amplification

Total RNA was extracted from macrophages, macrophages exposed to tumor cells or normal fibroblasts, tumor cells and normal fibroblasts using RNAagent (Total RNA Isolation System, Promega, Madison, WI). The RNA concentration was determined by a spectrophotometer reading at 260 and 280 nm. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using 4 U/μl of M-MLV reverse transcriptase (Promega). Aliquots of 5 μl of the cDNA were used for PCR amplification. The specific primers used for the identification of murine uPA, uPAR, MMP-2, MMP-9, β_2-microglobulin were: uPA (sense: 5′-TAT-GCA-GCC-CTA-TGG-CTC-3′; antisense: 5′-GAA-GTG-TGA-GAC-CCT-GTG-TAG-AC-3′, 210 bp product); uPAR (sense: 5′-GGA-CTC-CCA-AGG-CGG-CTG-CTG-C-3′; antisense: 5′-GGG-CCA-CCA-TTG-CAG-TGG-GTG-3′, 598 bp product); MMP-2 (sense: 5′-AGA-GAC-CTC-AGG-GTG-ACA-C-3′; antisense: 5′-AAG-AAG-TTG-TAG-TTG-GCC-A-3′, 330 bp product); MMP-9 (sense: 5′-GCT-CCT-GGC-TCT-CCT-GGC-TT-3′; antisense: 5′-GTC-CCA-CTT-GAG-GCC-TTT-GA-3′, 331 bp product) and β_2-microglobulin (sense: 5′-TGC-TAT-CCA-GAA-AAC-CCC-TC-3′; antisense: 5′-GTC-ATG-CTT-AAC-TCT-GCA-GG-3′, 258 bp product) [23]. All PCR reactions were performed using 0.1 U/μl of Go-Taq Polymerase (Promega). Amplification was carried out on a Perkin-Elmer Thermal cycler. Ten microliters of each PCR product were visualized after electrophoresis in a 2% agarose gel containing ethidium bromide 0.5 μg/ml. cDNA products were evaluated on the basis of a standard PCR marker (Promega).

Zymography

The gelatinolytic activity released by macrophages, macrophage:tumor cell co-cultures and tumor cells into their respective growth medium was tested by means of

electrophoresis on a 8% SDS-PAGE gels co-polymerized with 0.1% (w/v) type A gelatin (Sigma). Gels were washed for 30 min in 2.5% (v/v) Triton X-100 (Sigma) in order to remove SDS, and then incubated for 24 h at 37 °C in 50 mM Tris–HCl, pH 7.4, containing 200 mM NaCl and 5 mM $CaCl_2$. In order to visualize the zone of lysis, the gels were stained for 60 min at room temperature with 0.1% Coomassie brilliant blue (Merck), and destained for 2 h in a solution of methanol/acetic acid/water (3:1:6; v/v). The gelatinolytic activity was demonstrated as clear bands on a blue background. Moreover, the gelatinolytic activities of HT1080 human fibrosarcoma cells were used as markers of molecular weight.

Invasion assay

Invasiveness of F10-M3 melanoma cells was determined on Matrigel-coated polycarbonate filters (8 μm pore size) mounted in Boyden's chambers. The coated filters were prepared by using Matrigel suspensions at 250 μg/ml. 25×10^3 F10-M3 melanoma cells suspended in 200 μl DMEM 4500 containing 250 μg/ml BSA (DMEM/BSA) were seeded in upper chamber and incubated for 18–24 h. After incubation, cells on the upper side of the filters were wiped off and the membranes were fixed overnight in ice-cold methanol. Cells on the lower side of the membranes were stained with Diff Quick and counted.

Migration of F10-M3 murine melanoma cells was evaluated in cells grown in DMEM 4500 supplemented with FCS and then resuspended in: (a) a macrophage conditioned medium; (b) a medium conditioned by macrophage:tumor cell co-cultures; (c) a medium conditioned by macrophage:fibroblast co-cultures; (d) a medium conditioned by macrophage:tumor cell co-cultures supplemented with anti-MMP-9 monoclonal antibodies (17.5 μg/ml, final concentration) (AB19047, Chemicon, USA); and (e) a medium conditioned by macrophages co-cultivated with tumor cells in the presence of anti-uPAR monoclonal antibodies (26 μg/ml, final concentration) (MAB531, R&D System). F10-M3 murine melanoma cells grown in a standard medium, and then migrated in a fresh DMEM/BSA medium, were used as a control.

Western blotting analysis

Cells or macrophages were washed with ice cold PBS containing 1 mM Na_4VO_3, and lysed in 100 μl of cell lysis buffer containing: 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 100 mM HEPES, 1% Triton X-100, 10 mM glycerophosphate, 100 μM PMSF, 100 mM AEBSF, 5 mM bestatin, 2 mM leupeptin, 1 mM pepstatin A, and 80 μM of aprotinin, 1.5 mM E-64. Aliquots of supernatants containing equal amounts of protein (25 μg) in Laemmli buffer were separated on 8% (v/v) SDS-PAGE gel. Fractionated proteins were transferred from the gel to a PVDF nitrocellulose membrane using an electroblotting apparatus (Bio-Rad). Blots were stained with Ponceau red to ensure equal loading and complete transfer of proteins, and then blocked for 1 h, at room temperature, with Odyssey blocking buffer (M-Medical). Subsequently, the membrane was probed at 4°C overnight with goat anti-mouse uPAR monoclonal antibodies (R&D Systems) diluted in a solution of 1:1 Odyssey blocking buffer/T-PBS buffer. The membrane was washed in T-PBS buffer, incubated for 1 h at room temperature with a rabbit anti-goat Alexa Flour 680 antibodies (Invitrogen) (dilution 1:7,000 in Odyssey blocking buffer), and then visualized by an Odyssey Infrared Imaging System (LI-COR Bioscience). Rabbit anti-murine β-actin (Santa Cruz) monoclonal antibodies, diluted 1:10000 in T-PBS, were used to assess equal amount of protein loaded in each lane.

Statistical analysis

The statistical significance of the differences was determined using Student's t-test, and defined a $p < 0.05$. Invasion assays were done in triplicate in at least two-three different experiments. Data are expressed as means ± SEM.

Results

Change of invasiveness and protease expression in IFNγ-stimulated F10-M3 murine melanoma cells

Figure 1 (panel a) shows that 100 U/ml of IFNγ stimulated invasiveness of F10-M3 murine melanoma cells through Matrigel-coated filters. Moreover, treatment of IFNγ-stimulated tumor cells with JAK inhibitor-I or with AG490, two well known inhibitors of JAK1 and JAK2 signal transduction factors [24, 25], prevented the pro-invasive activity of IFNγ on cell invasion. As shown in Fig. 1 (panel b), 100 U/ml of IFNγ stimulated mRNA expression for uPAR and MMP-9 in melanoma cells. The increase of invasiveness through Matrigel of IFNγ-stimulated melanoma cells was prevented in the presence of a migration medium supplemented with anti-uPAR or anti-MMP-9 monoclonal antibodies (Fig. 1, panel c).

Change of MMPs and uPA/uPAR expression in thioglycollate-elicited macrophages after co-cultivation with syngeneic F10-M3 melanoma cells

As shown in Fig. 2 (panel a), thioglycollate-elicited macrophages expressed higher levels of mRNAs for uPA,

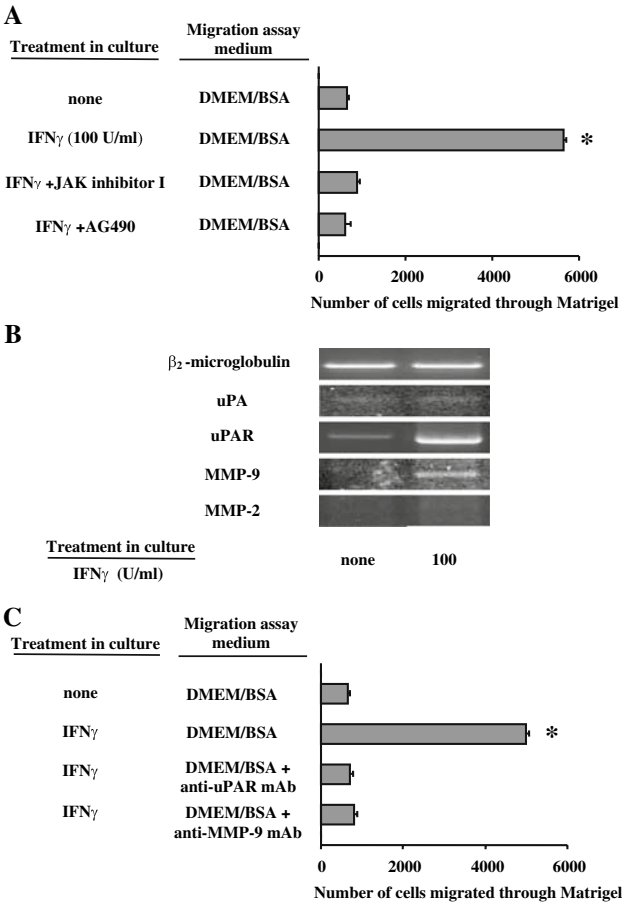

Fig. 1 Abrogation of invasiveness in IFNγ-stimulated F10-M3 murine melanoma cells challenged with anti-uPAR or anti-MMP-9 monoclonal antibodies. (**a**) Invasiveness of tumor cells stimulated with 100 U/ml of IFNγ in the presence of JAK inhibitor I (500 nM) or AG490 (500 nM). (**b**) RT-PCR analysis of mRNA for uPA/uPAR and MMPs in tumor cells stimulated with 100 U/ml of IFNγ(representative gel of 3–4 independent experiments). (**c**) Change in invasiveness in IFNγ-stimulated tumor cells re-suspended in a migration medium (DMEM/BSA) containing anti-uPAR or anti-MMP-9 monoclonal antibodies. *Significantly different from cells grown in a standard medium and migrated in DMEM/BSA, at $p < 0.05$

uPAR, MMP-9 and MMP-2 following stimulation with IFNγ/LPS, a behaviour reflecting a state of "responsive" macrophages [26]. Macrophages expressed higher levels of mRNAs for uPAR and MMP-9, once co-cultivated with F10-M3 murine melanoma cells, and an higher pro-MMP-9 activity was found in the growth medium of macrophage: tumor cell co-cultures (Fig. 2, panel b). The higher level of mRNA for uPAR found in macrophages co-cultivated with tumor cells was also accompanied by an increased level of uPAR protein (Fig. 3, panel c). The up-regulation of uPAR and MMP-9 expression in macrophages co-cultivated with tumor cells seemed to be a phenomenon independent of soluble factors secreted by melanoma cells, but rather related to macrophage/melanoma cell contact. In fact, we did not find any change in mRNA expression for uPAR and MMP-9 in macrophages either cultivated in transwell plates with F10-M3 melanoma cells, or grown in a medium conditioned by melanoma cells themselves (Fig. 2, panel d). Furthermore, the promoting activity of melanoma cells on the expression of macrophage proteases appeared to be rather specific, in view of the finding that macrophages cultivated with normal murine fibroblasts expressed the same low levels of mRNA for uPAR and MMP-9 as those shown by macrophages grown in a standard medium (Fig. 2, panel e).

Change of invasiveness in F10-M3 murine melanoma cells exposed to a medium conditioned by macrophage: tumor cell co-cultures

As shown in Fig. 3 (panel a), F10-M3 murine melanoma cells became more invasive through Matrigel when they were tested in the presence of a medium conditioned by macrophage: tumor cell co-cultures, an effect that was abrogated by anti-MMP-9 monoclonal antibodies. On the other hand, the invasiveness through Matrigel was unchanged in melanoma cells tested in the presence of a medium conditioned by macrophage: fibroblast co-cultures, both in the absence or in the presence of anti-MMP-9 monoclonal antibodies. As shown in Fig. 3 (panel b), a medium conditioned by macrophages co-cultivated with melanoma cells in the presence of anti-uPAR monoclonal antibodies for the entire period of co-cultivation failed to enhance the invasiveness of melanoma cells. This finding ruled out the possibility that uPA system stimulates MMP-9 activation in macrophages grown together with tumor cells. Western blotting analysis showed an increase extracellular-signal regulated kinase (ERK1/2) in macrophages co-cultivated with tumor cells, that was abrogated by mAb anti-uPAR (see box in Fig. 3, panel b).

Discussion

The present study shows that IFNγ-stimulated invasiveness of F10-M3 murine melanoma cells is associated with a greater expression of uPAR and MMP-9. Moreover, the IFNγ-stimulated invasiveness was blocked by treatment with monoclonal antibodies anti-uPAR or anti-MMP-9, suggesting a role of uPA and MMP-9 in the pro-invasive activity of IFNγ. A cooperation between uPA system and MMP-9 was reported for intravasation of cancer cells of a different origin [27]. In addition, the invasion and metastatic diffusion of glioblastoma cells was prevented by the use of small interfering RNA expression vectors for uPAR and MMP-9 [28]. These results are in agreement with the finding that patients with pancreatic tumors expressing

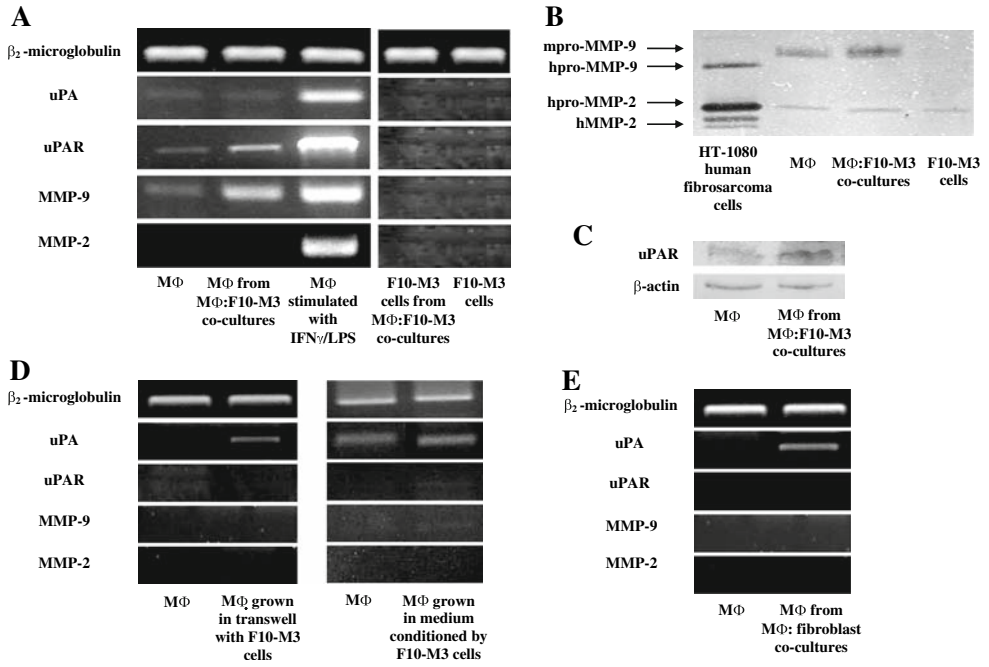

Fig. 2 uPA, uPAR, MMP-9 and MMP-2 expression in thioglycollate elicited-macrophages (MΦ) co-cultivated with F10-M3 murine melanoma cells, normal fibroblasts or grown in a medium conditioned by tumor cells themselves. (**a**) mRNA expression for uPA, uPAR, MMP-9 and MMP-2 in macrophages co-cultivated with tumor cells, in macrophages stimulated in vitro with IFNγ/LPS, and in F10-M3 melanoma cells grown in standard conditions or co-cultivated with macrophages. (**b**) Gelatin zymogram of a medium conditioned by macrophages cultivated in the absence or in the presence of melanoma cells. (**c**) Western blotting analysis of uPAR in macrophages grown with tumor cells. (**d**) mRNA expression for uPA, uPAR, MMP-9 and MMP-2 in thioglycollate-elicited macrophages grown with F10-M3 murine melanoma cells in transwell plates or cultured in a medium conditioned by tumor cells themselves. (**e**) mRNA expression for uPA, uPAR, MMP-9 and MMP-2 in thioglycollate elicited-macrophages co-cultivated with normal murine skin fibroblasts. (Representative gels of 4–5 independent experiments)

high levels of uPA and MMP-9 had a shorter survival time [29].

The present study also shows that murine melanoma cells expressed a greater invasiveness through Matrigel, when migration assay was performed in the presence of a medium conditioned by macrophages co-cultivated with tumor cells. This phenomenon was found to be associated with an increased expression of uPAR and MMP-9 in macrophages isolated from tumor cells. However, by the use of monoclonal antibodies we found that the enhanced invasiveness of melanoma cells exposed to a medium conditioned by macrophage: tumor cell co-cultures was dependent on MMP-9, but not on uPAR. Preliminary experiments showed that other protease activities secreted by macrophages and/or tumor cells are implicated in the activation of MMP-9 in macrophages co-cultivated with melanoma cells. Indeed, a medium conditioned by macrophages co-cultivated with tumor cells in the presence of a cocktail of inhibitors for aspartic, cysteine and serine protease, failed to stimulate invasiveness through Matrigel in melanoma cells (data not shown). In view of the signal transduction functions of uPAR [5], we verified whether the enhanced expression of uPAR in macrophages co-cultivated with tumor cells was associated with a change of ERK. By the use of a anti-uPAR mAb, we found an association between the enhanced expression of uPAR and an enhancement of ERK1/2 in macrophages co-cultivated with melanoma cells. uPAR and MMP-9 expression in macrophages co-cultivated with tumor cells seems a rather specific phenomenon, probably evoked by a cell-to-cell contact mechanism. Histopathological studies revealed a tight contact between macrophages and tumor cells at the invasive edges of tumors [30, 31].

The contribution of stromal-derived MMPs to melanoma progression was supported by the observation that injection of B16 melanoma cells into the tail vein of MMP-2 and MMP-9 null mice led to a significant decrease in the number of lung metastases compared to that found in mice expressing MMPs [32]. Moreover, a lower incidence of tumors was found in the peritoneal cavity of immunodeficient MMP-9 null mice transplanted with human ovarian cancer cells [33]. It has been reported that MMP-9-expressing TAMs promote secondary organ colonization of human and murine tumor cells [34].

On the whole, our data revealed that uPAR and MMP-9 are generated by cytokine-stimulated murine melanoma

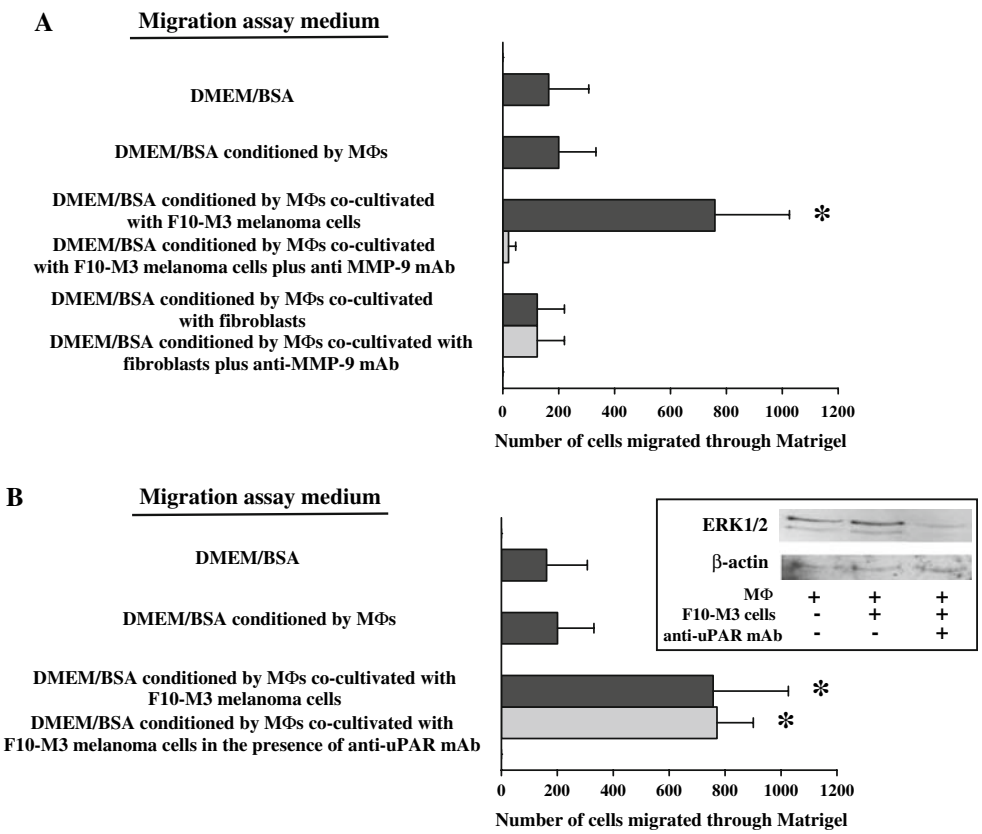

Fig. 3 Change of invasiveness in F10-M3 murine melanoma cells migrated in: (**a**) a medium (DMEM/BSA) conditioned by macrophage: tumor cell co-cultures in the presence of anti-MMP-9 monoclonal antibodies; and (**b**) a medium (DMEM/BSA) conditioned by macrophages co-cultivated with tumor cells in the presence of anti-uPAR monoclonal antibodies. See in box of panel b the change of ERK1/2 in macrophages co-cultivated with F10-M3 murine melanoma cells in the presence or in the absence of anti-uPAR mAb. *Significantly different from cells migrated in DMEM/BSA at $p < 0.05$

cells as well as macrophages elicited by melanoma cells themselves, as a result of the reciprocal interaction between the two types of cells. This finding suggests that redundant mechanisms operating in the tumor inflammatory micro-environment are responsible of the transition of tumor cells to an invasive phenotype. Thus, beside tumor cells, host inflammatory cells should also be considered as targets in the therapeutic approach of cancer.

Acknowledgements This work was supported by grants from Murst ex 60% and Ente Cassa di Risparmio di Firenze.

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