Cytokine-dependent invasiveness in B16 murine melanoma cells: Role of uPA system and MMP-9

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Abstract. Proteases are crucial for the spread of cancer cells from a primary tumor to the site of secondary growth. This study examined the ability of IFNγ and TNFα to stimulate a better invasiveness in B16 murine melanoma cells, and investigated whether this enhanced ability was related to a higher expression of protease activities, such as urokinase plasminogen activator (uPA) and its receptor (uPAR), and matrix metalloproteinases 2 and 9 (MMP-2, MMP-9). We found that murine melanoma cells enhanced their lung-colonizing potential *in vivo* and invasiveness through Matrigel-coated filters upon costimulation with IFNγ and TNFα; neither IFNγ nor TNFα alone, at the dose used in the experiments, was able to elicit a change in the invasive/metastatic efficiency of melanoma cells. The invasive phenotype of murine melanoma cells stimulated with IFNγ and TNFα was characterized by an enhanced uPA/uPAR and MMP-9 expression: TNFα promoted MMP-9 mRNA expression and pro-MMP-9 protein secretion, and the costimulation with IFNγ and TNFα was required to potentiate the expression of mRNA and protein for uPAR, and to induce a redistribution of uPA from the soluble to the cell body-associated form. Both monoclonal antibodies, anti-uPAR and anti-MMP-9, caused a significant reduction of invasiveness in IFNγ/TNFα-stimulated melanoma cells. These results indicate that invasiveness in B16 murine melanoma cells can be regulated in a cytokine-specific fashion and is dependent on the synergism between the uPA/uPAR system and MMP-9.

Introduction

Experimental evidence has led to the recognition that the expression in tumor cells of some biological activities typical of a metastatic phenotype is due to temporary genetic changes caused by cellular and humoral host factors (1,2). The tumor microenvironment contains many host cells, such as mast cells, fibroblasts, endothelial cells, macrophages, and lymphocytes. Among the various types of host cells, tumor-associated macrophages (TAMs) play a critical role in tumor progression. Whereas TAMs may destroy tumor cells following their activation (3,4), they may also produce growth factors, cytokines and proteases that promote invasion and metastatic diffusion of tumor cells (5-13). It is of particular interest that the failure to recruit macrophages into the tumor microenvironment in colony stimulating factor-deficient (CSF^{op}/CSF^{op}) mice reduced the metastatic spread of transplanted mammary cancer (14). Studies performed in our laboratory showed that macrophages, elicited by immune agents (*C. parvum*, BCG, *L. monocytogenes*), secreted in their growth medium a pro-metastatic activity which enhanced the ability of B16 murine melanoma cells to colonize the lung of syngeneic mice (10) and migrate through Matrigel (15). We demonstrated that this macrophage prometastatic activity had to be ascribed to the cooperative effect of two inflammatory cytokines, IFNγ and TNFα. The capacity of exogenous IFNγ to stimulate the formation of metastases in murine melanoma cells was related to the capacity of IFNγ to induce a higher expression of MHC class I antigens (17-19), a change to escape NK activity (17,18). IFNγ may also promote metastatic diffusion of melanoma cells through a down regulation of the processing of tumor antigens (20-22). TNFα promoted the invasiveness of human melanoma cells through the stimulation of the expression of integrin receptors (23), or cell-bound degradative enzymes (24).

In this study, we explored whether the invasive/metastatic phenotype of B16 murine melanoma cells stimulated with exogenous IFNγ and TNFα was associated with a change in protease expression. Among the different proteases that might play a role in tumor dissemination, we focused our attention on matrix metalloproteinases 2 and 9 (MMP-2, MMP-9) and urokinase-type plasminogen activator (uPA) and its receptor (uPAR). Both systems of proteases are markers of melanoma progression. Indeed, uPA/uPAR and MMP are expressed in advanced stages of primary and metastatic melanoma lesions (25-27). Moreover, antisense oligonucleotide-driven inhibition of uPAR expression in human melanoma cells inhibited lung metastases in an experimental model of spontaneous metastasis in nude mice (28). Similar data are available for MMPs (26,27), whose control by genetic (29) and pharmacological means (30) has

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Table I. Oligonucleotide primer pairs used in RT-PCR analysis.

Gene product	Forward primer (5'→3')	Reverse primer (5'→3')	Product (bp)	Annealing temperature (˚C)	Cycles	Author/Ref.
uPA	TATGCAGCCCTATGGCTC	GAAGTGTGACCCTGTGTAGAC	210	60	34	Rakic, *et al* (41)
uPAR	GGACTCCCAAGGCGGCTGCTGC	GGGCCACCATTGCAGTGGGTG	598	55	34	Gutierrez, *et al* (42)
MMP-9	GCTCCTGGCTCTCCTGGCTT	GTCCCACTTGAGGCCTTTGA	331	55	30	Itoh, *et al* (29)
MMP-2	AGAGACCTCAGGGTGACAC	AAGAAGTTGTAGTTGGCCA	330	55	30	Itoh, *et al* (29)
$ß_2$ Microglobulin	TGCTATCCAGAAAACCCCTC	GTCATGCTTAACTCTGCAGG	258	55	30	Orlic, *et al* (43)

been shown to inhibit the aggressiveness of experimental melanoma. However, information on the control of these proteases by inflammatory cytokines in tumor cells is still limited, and an inconsistency in the trend is seen in the present information (31-38).

Materials and methods

Cell lines and culture conditions. In this study, we used a low metastatic clone of B16-F10 murine melanoma cells, the F10-M3 cells (39). Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM 4500, Gibco) supplemented with 10% fetal calf serum (Boehringer Mannheim, Germany), at 37˚C in a 10% CO_2 humidified atmosphere. Cells were harvested from subconfluent cultures by incubation with a trypsin-EDTA solution, and propagated every three days. Cell viability was determined by trypan blue exclusion test. Cultures were periodically monitored for mycoplasma contamination using Chen's fluorochrome test (40).

Cytokine-treated murine melanoma cells were obtained by incubating tumor cells, for a period of 24 h, in media supplemented with IFNγ (25 U/ml) and/or TNFα 25 ng/ml) (Peprotech, England). Cytokine concentrations were chosen based on our previous experiments performed to investigate the prometastic activity of IFNγ and TNFα (16).

Lung colonization. B16 melanoma cells were harvested from dishes by the use of trypsin-EDTA solution. Cells were washed twice in PBS and then suspended in serum-free medium at $25x10^5$ cells/ml. 0.2 ml of this suspension was injected into the tail veins of syngeneic C57Bl/6 mice. The animals were monitored and sacrificed 21 days later. The lungs were inspected using a dissecting microscope and lung nodules were counted.

In vitro invasion assay. Invasiveness of B16 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. B16 melanoma cells ($25x10^3$) suspended in 200 μl DMEM 4500 containing 250 μg/ml BSA were seeded in the 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.

In some experiments, the migration ability of tumor cells was tested in the presence of monoclonal antibodies anti-uPAR (26 μg/ml, final concentration) (R&D Systems, USA), and monoclonal antibodies anti-MMP-9 (17.5 μg/ml, final concentration) (Chemicon, USA).

RNA isolation and polymerase chain reaction (PCR). Total RNA was extracted from cells using Trizol reagent (Gibco), and the amount and purity of the RNA was determined spectrophotometrically. cDNA was synthesized from 1 μg of total RNA using 4 U/μl of M-MLV reverse transcriptase (Promega, Madison, WI). Aliquots of 5 μl of the cDNA were used for PCR amplification. The murine uPA, uPAR, MMP-2, MMP-9, $ß_2$-microglobulin were identified using the primers listed in Table I. All PCR reactions were conducted using 0.1 U/μl of Go-Taq polymerase (Promega). Amplification was carried out on a Perkin-Elmer thermal cycler. Ten μl of each PCR product were visualized after electrophoresis in a 2% agarose gel containing 0.5 mg/ml of ethidium bromide. cDNA products were evaluated on the basis of a standard PCR marker (Promega).

Western blotting analysis. B16 melanoma cells were washed twice with ice-cold PBS containing 1 mM Na_4VO_3. Cells were 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. Supernatants containing equal amounts of protein (65 μg) in Laemmli buffer were separated on 10% (v/v) SDS-PAGE gel. Fractionated proteins were transferred from the gel to a PVDF nitrocellulose membrane. Blots were stained with Ponceau red to ensure equal loading and complete transfer of proteins and then blocked with 2.5% (w/v) bovine serum albumin (BSA) in T-TBS [0.1% (v/v) Tween-20 in PBS] for 1 h at room temperature. Subsequently, the membrane was probed with uPAR (R&D Systems) goat anti-mouse monoclonal antibody, 1:1000 diluted with 2.5% (w/v) skimmed milk in T-PBS buffer at 4˚C overnight. After washing in T-PBS buffer, the membrane was incubated with a horseradish peroxidase-conjugated rabbit anti-goat antibody (Santa Cruz, USA), 1:8000 dilution in 2.5% (w/v) BSA/T-PBS, for 1 h. After successive washing with T-PBS, the immunoreactive bands were visualized using the ECL detection system (Amersham, Arlington Heights, IL, USA).

Zymography for MMPs. Aliquots from media conditioned by tumor cells were electrophoresed on 8% SDS-polyacrylamide gels co-polymerized with 0.1% (w/v) type A gelatin (Sigma). After electrophoresis, the gels were washed in 2.5% v/v Triton X-100 (Sigma) for 30 min to remove SDS. Gelatin substrate gels were then incubated in 50 mM Tris-HCl, pH 7.4, 200 mM NaCl and 5 mM $CaCl_2$ for 24 h at 37˚C. After incubation, the gels were stained with 0.1% Coomassie brilliant blue (Merck) in acetic acid, methanol and distilled water at a volume ratio of 1:2:3, respectively, for 60 min at room temperature. The gels were destained in the same solution without the Coomassie for 2 h. After destaining, the gels were immersed in distilled water and scanned immediately. Gelatinolytic activity was demonstrated as clear bands on a blue background.

Analysis of uPA enzymatic activity. Culture medium and cell lysate aliquots were concentrated by centrifugation at 8000 rpm for 30 min in centricon tubes (Amicon Division, Beverly, MA, USA) with 30-kDa molecular weight cut-off pores. The samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis (10%) under non-reducing conditions and migrated proteins were transferred onto a 0.45-μm pore-size nitrocellulose filter (BioRad Laboratories, Richmond, California, USA) in a 40-mM phosphate buffer (pH 6.5), under a current of 0.4 A for a 2-h run. The nitrocellulose filter was removed and placed on an indicating layer containing casein and plasminogen. After overnight incubation at 37˚C, when clear bands of lysis were visible in the cloudy casein background, corresponding to the position of plasminogen activators in the polyacrylamide gel, zymograms were dried and stained with Ponceau S solution (Sigma). uPA activity was measured by densitometric scanning of the zymograms, against standard uPA.

Statistical analysis. The statistical significance of the differences between unstimulated tumor cells and cytokine-stimulated tumor cells was determined using the Student's t-test. Invasion assays were performed in triplicate in at least two to three different experiments. Data are expressed as means ± SEM.

Results

Fig. 1A shows that the combined treatment of B16 murine melanoma cells with IFNγ and TNFα promoted a better capacity to colonize lungs of syngeneic animals, an effect which neither IFNγ nor TNFα alone, as reported in a previous paper by our laboratory (16), was able to elicit. B16 murine melanoma cells stimulated with IFNγ and TNFα also showed a greater invasiveness through Matrigel-coated filters (Fig. 1B).

RT-PCR analysis and zymography revealed that melanoma cells, grown in standard conditions, do not express MMP-2 and MMP-9, a behaviour which also characterized melanoma cells treated with IFNγ (Fig. 2). At the variance with this behaviour, TNFα and the combination of IFNγ and TNFα, stimulated MMP-9 mRNA expression in melanoma cells (Fig. 2A). Zymography of media conditioned by stimulated tumor cells revealed that TNFα and the combination of IFNγ and TNFα stimulated pro-MMP-9 release (Fig. 2B). As shown in Fig. 3, IFNγ and TNFα promoted the expression of uPAR

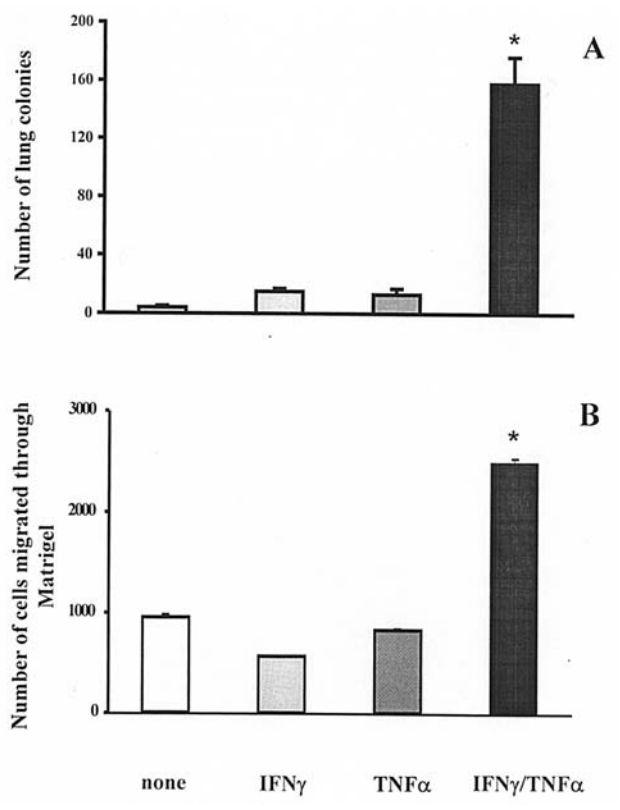

Figure 1. Change in lung colonization (A) and invasiveness through Matrigel (B) in F10-M3 murine melanoma cells stimulated with IFNγ and/or TNFα. *Significantly different at p<0.001.

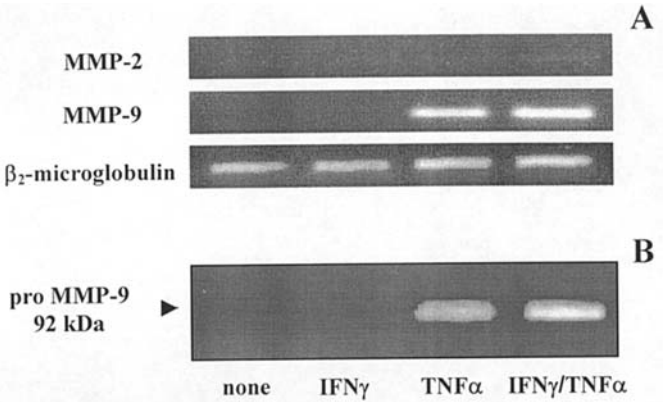

Figure 2. Change in MMP-9 in F10-M3 murine melanoma cells stimulated with IFNγ and/or TNFα. RT-PCR analysis of total RNA for MMP-2 and MMP-9 (A), and gelatin zymogram of conditioned media (B) of tumor cells grown in the presence and absence of cytokines.

mRNA (panel A) and protein (panel B) in tumor cells. IFNγ alone induced a slight increase of mRNA for uPAR (panel A) that was not associated with a change in protein synthesis (panel B). The level of mRNA for uPA was found to remain unchanged in tumor cells stimulated with IFNγ and/or TNFα (panel A) but, as revealed by casein zymography of media conditioned by tumor cells costimulated with IFNγ and TNFα performed in parallel with casein zymography of cell bodies of the same tumor cells, uPA redistributed from the soluble to the cell body-associated compartment (panel C).

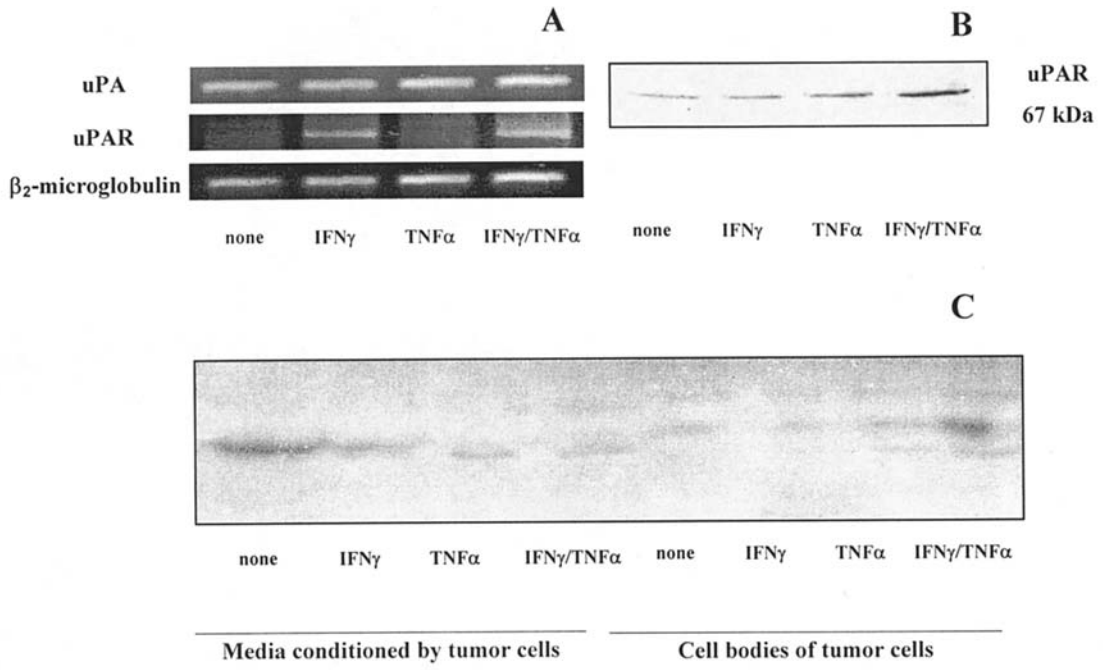

Figure 3. Change in uPA/uPAR expression in F10-M3 murine melanoma cells stimulated with IFNγ and/or TNFα. RT-PCR analysis of total RNA for uPAR and uPA (A), Western blotting of uPAR (B), and casein zymogram of conditioned media and cell bodies (C) of tumor cells grown in the presence and absence of cytokines.

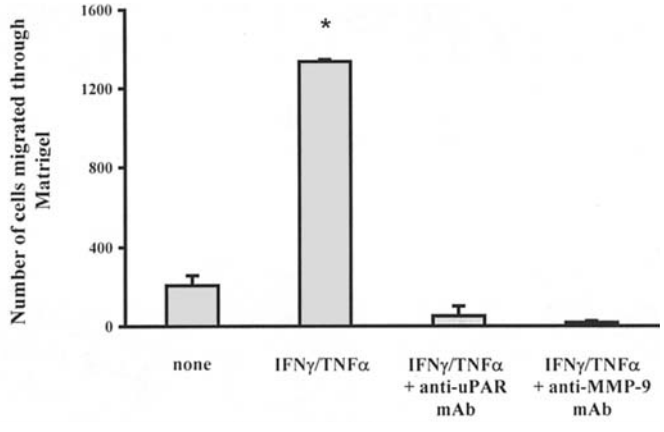

Figure 4. Invasiveness of F10-M3 murine melanoma cells costimulated with IFNγ and TNFα and with IFNγ and TNFα and anti-uPAR or anti-MMP-9 monoclonal antibodies (mAb). *Significantly different at $p<0.001$.

Fig. 4 shows that both anti-uPAR and anti-MMP-9 monoclonal antibodies abrogated the enhanced invasiveness of melanoma cells costimulated with IFNγ and TNFα.

Discussion

An important feature of metastatic cells is their ability to escape from their site of origin by invading surrounding tissue, including basement membranes. The present study was aimed at clarifying whether IFNγ/TNFα-dependent invasive/metastatic phenotype in B16 murine melanoma cells was associated with an enhanced expression of uPA/uPAR and MMP protease activity. We found that the high lung-colonizing potential, as well as the better invasiveness through Matrigel-coated filters, expressed by murine melanoma cells costimulated with IFNγ and TNFα was associated with an overexpression of uPA/uPAR and MMP protease activity. In particular, TNFα accounted for a higher expression of MMP-9 mRNA and pro-MMP-9 protein secretion, and the cooperation between IFNγ and TNFα was responsible for a greater expression of uPAR mRNA and protein, leading to the enrichment of uPA on tumor cell membrane. It is possible that the cytokine-potentiation of invasiveness in B16 melanoma cells might be related to the activation of a program that implies an enhanced expression of uPAR, thereby favouring the partitioning of uPA on its receptor and initiation of a protease cascade which also includes pro-MMP-9 activation. The inhibition of the enhanced invasiveness of tumor cells costimulated with IFNγ and TNFα by anti-uPAR and anti-MMP-9 monoclonal antibodies sustained the notion that the increased migration of melanoma cells was dependent on the cooperation between uPA/uPAR and MMP-9, and that there was not a hierarchy of a single system. In light of the co-expression by tumor cells of these two protease systems, Lakka *et al* (44) reported that a single vector capable of expressing small interfering RNA for uPAR and MMP-9 can block the expression of targeted proteases in glioblastoma cells, and consequently invasion, tumor growth and angiogenesis. Cooperation between uPA/uPAR and MMP-9 was found to be required for intravasation, a rate-limiting step for metastatic diffusion, of different types of cancer cells (epidermoid carcinoma cells, breast cancer cells, prostate cancer cells, fibrosarcoma cells) (45). Moreover, the evaluation of uPA/uPAR and MMP-9 in pancreatic cancer cells led to the conclusion that these proteases are potential prognostic indicators, e.g. patients with overexpression of uPA and MMP-9 in their tumor cells have a trend toward a shorter survival time (46). MMP-9 is not the only member of the

MMP system which has been shown to be functionally coupled with uPA/uPAR, as cooperation between the uPA system and MMP-2 was found by Ellenrieder *et al* (36) in the enhanced invasiveness of pancreatic cancer cells stimulated with TGF-ß. Further clarification of the mechanisms by which uPA/uPAR and MMP-9 operate in tumor cells will be important, in view of evidence suggesting new functions for these proteases in cancer progression, e.g. adhesion, growth and apoptosis, angiogenesis and immune response (47-50).

In conclusion, the data reported in this study suggest that the striking invasive phenotype exhibited by B16 murine melanoma cells costimulated with IFNγ and TNFα was dependent on cooperation between the uPA/uPAR system and MMP-9 activity, a finding that might offer a novel therapeutic strategy to combat dissemination of tumor cells, e.g. targeting the extracellular mediators that work at the tumor-host communication interface.

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