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Integrin β3 cDNA Transfection into a Highly Metastatic αvβ3-Negative Human Melanoma Cell Line Inhibits Invasion and Experimental Metastasis

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Even though integrin αvβ3 is thought to play a role in invasive growth of melanomas, some metastatic melanoma cell lines lack αvβ3, and downmodulation of αvβ3 expression can enhance the invasive capacity of certain melanoma cells. To further investigate this apparent dualistic role of αvβ3, we transfected β3 cDNA into the highly metastatic, β3-negative human melanoma cell line MV3. MV3 cells adhered to fibronectin but not to fibrinogen or a synthetic RGD peptide, while MV3-β3 adhered to all three RGD-containing adhesive ligands, and this adhesion was inhibited by LM609 αvβ3 mAb. Expression of αvβ3 did not affect MV3 *in vitro* proliferation or *in vivo* tumorigenicity upon subcutaneous inoculation into nude mice. In contrast, it strongly reduced invasion in matrigel and lung colonization in nude mice of MV3 cells. Thus, certain melanoma cell lines have adopted a metastatic strategy in the absence of αvβ3, and in such cells expression of this integrin leads to a less aggressive phenotype.

Integrins are heterodimeric transmembrane receptors that mediate adhesion of cells to other cells and to the extracellular matrix (ECM) [1]. Not only is integrin-mediated adhesion required for cell migration [2] but it also generates signals that affect proliferation and differentiation of cells [3,4]. Both phenomena implicate integrins in tumor metastasis.

Several studies suggest that integrin αvβ3 plays an important role in invasive growth of melanoma cells [5]. Conversely, highly metastatic human melanoma cell lines have been described that lack αvβ3 expression [6,7], and for certain melanoma cells downmodulation of expression of αvβ3 actually results in increased invasiveness [8]. Therefore, in the present study, we transfected β3 cDNA into the highly metastatic, αvβ3-negative human melanoma cell line MV3, and studied its effect on growth, invasion, and experimental metastasis.

MATERIALS AND METHODS

ECM proteins and antibodies. Human plasma fibrinogen (Fg) and fibronectin (Fn) were from Sigma (St Louis, MO). A synthetic Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) peptide was prepared and coupled to BSA as described [9]. Anti-integrin mAbs included 4B4 anti-β1 [10], from Coulter Immunology (Hialeah, FL); LM142 anti-αv and LM609 anti-αvβ3 [11], from Dr. David Cheresh (La Jolla, CA); and P1F6 anti-αvβ5 [12] from Life Technologies (Gaithersburg, MD).

Nude mice and cell culture. BALB/c athymic nude mice (nu/nu) from The Laboratory Breeding and Research Center (Bomholtgaard, Ry, Denmark) were kept in cages covered with air filters under specific pathogen-free conditions and used when 6-8 weeks old. Within a single experiment mice were sex and age matched. MV3 human melanoma cells [13] were cultured in Dulbecco's modified Eagles medium (DMEM; Flow, Irvine, UK), supplemented with 10% fetal bovine serum (Life Technologies) and antibiotics.

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Abbreviations: ECM, extracellular matrix; Fg, fibrinogen; FITC, fluorescein isothiocyanate; Fn, fibronectin; mAb, monoclonal antibody; i.v., intravenous; MTT, 3-[4,5-dimethylthiazol]-2,5-diphenyl tetrazolium bromide; s.c., subcutaneous.

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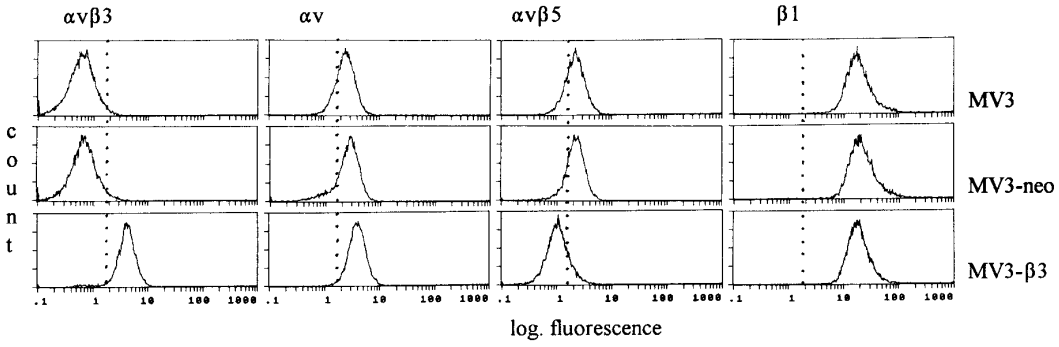

FIG. 1. Integrin expression in MV3 transfectants. MV3 cells were either untransfected or transfected with pBJ1neo (MV3-neo), or with pBJ1neo including β3 cDNA followed by bulk sorting with LM609 $\alpha v\beta 3$ mAb (MV3-β3). Shown is the relative fluorescence after incubation with mAbs to integrin subunits as indicated and FITC-labeled second antibody.

Transfection. Full-length cDNA for the integrin β3 subunit [14], from Dr. Erkki Ruoslahti (La Jolla, CA), was cloned in the polylinker of the mammalian expression vector pBJ1neo [15], from Dr. Rene de Waal-Malefijt (La Jolla, CA). Twenty μg of this construct was used for stable transfection of MV3 cells according to the calcium phosphate precipitation method [16]. Stably transfected cells were selected in the presence of 1 mg/ml G418 (Life Technologies) and bulk sorted with LM609 mAb on an Epics flowcytometer (Coulter, Mijdrecht, The Netherlands).

Flow cytometry. Cells were incubated sequentially with mAbs and fluorescein-isothiocyanate (FITC)-labeled $F(ab')_2$ fragments of rabbit anti-mouse Ig antibodies (Dako, Glostrup, Denmark) for 30 min at 4°C, and fluorescence was measured on an Epics flowcytometer (Coulter).

Cell adhesion assay. Polystyrene microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated overnight at 4°C with the appropriate adhesive ligands, and blocked for 1h at 37°C with 0.5% BSA. Subsequently, 1×10^4 [^{51}Cr]-labeled MV3 cells in 50 μl DMEM/0.5% BSA were added to the wells and incubated for 30 min at 37°C in 5% CO_2. Unbound cells were washed out, bound cells were lysed, and radioactivity of the lysate was measured. Results are presented as the mean percentage of cell binding from triplicate wells. For adhesion inhibition studies, cells were preincubated with the appropriate mAbs for 30 min at 4°C before seeding into the wells.

Invasion assay. Matrigel-coated 8μm pore filters (24 wells; Becton Dickinson, Bedford, MA) were preincubated with serum-free medium for 2 h at 37°C. Subsequently, 900 μl complete medium was added to the lower compartment and 1×10^5 cells in 200 μl serum-free medium were added to the upper compartment. Filters were incubated for 48h at 37°C. For visual inspection, filters were fixed in 2% glutaraldehyde, cells were scraped from the upper surface, filters were washed with PBS, incubated for 15 min in Mayers hematoxylin, washed, and scored by light microscopy. For semiquantitative analysis [17], cells were scraped from the upper surface, filters were washed with PBS, and incubated for 4h at 37°C in 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid (MTT; Sigma). Subsequently, purple formazan crystals formed in active mitochondria were solubilized in 10% SDS in 0.01 M HCl o/n at 37°C. Absorbance of the solutions was measured at 540 nm with a 690 nm reference wavelength.

Tumorigenicity and experimental metastasis assay. For tumorigenesis, 2×10^6 tumor cells in 200 μl 0.9% NaCl were subcutaneously (s.c.) inoculated and tumor volume was measured weekly. For lung colonization, 2×10^6 tumor cells in 200 μl 0.9% NaCl were intravenously (i.v.) inoculated into the lateral tail vein, mice were killed after 1 month, lungs were formalin fixed and embedded in paraffin, and H&E-stained 4 μm sections from 3 different levels of the lungs were microscopically examined for colonies. In our experience, no other organs are colonized [13].

Statistical analysis. Fisher's Exact Test (two-sided) was used for comparison of percentages of mice that developed metastases.

RESULTS

MV3 did not express $\alpha v\beta 3$ but it expressed other αv integrins including $\alpha v\beta 5$ (Fig 1). The expression pattern was not altered in MV3-neo whereas MV3-β3 expressed $\alpha v\beta 3$ at the surface. Total αv-staining was not affected but the level of $\alpha v\beta 5$ was decreased, indicating that β3 competed with β5 for association with the αv subunit. No changes were observed in the level of expression of β1 (Fig 1) or any other integrin subunit (including α1-6, αIIb, β2, β4, β8; not shown). MV3-β3 but not MV3 or MV3-neo adhered to Fg- or GRGDSP-coated

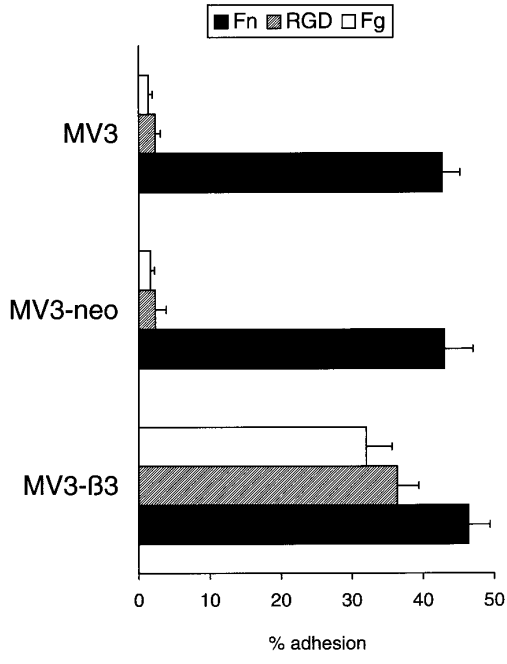

FIG. 2. Effect of $\alpha v\beta 3$ on MV3 adhesion. MV3, MV3-neo, or MV3-$\beta 3$ cells were allowed to adhere to wells coated with 25 μg/ml Fg, BSA-GRGDSP or Fn. Mean $\pm$ s.d. from triplicate determinations is shown for 1 experiment of 3.

wells while all 3 cell lines adhered to Fn (Fig 2). The acquired adhesion of MV3-$\beta 3$ to Fg and the RGD peptide was inhibited 62% and 54% respectively by the LM609 anti-$\alpha v\beta 3$ mAb while P1F6 anti-$\alpha v\beta 5$ had no effect (Fig 3).

In vitro proliferation rates of MV3, MV3neo, and MV3-$\beta 3$ were identical (not shown). Upon s.c. inoculation into nude mice, all 3 cell types were equally effective in tumor formation and growth (Fig 4). In contrast, in an in vitro matrigel invasion assay, a reduced number of MV3-$\beta 3$ cells were detected on the bottom of filters compared to MV3 and MV3-neo. By using the MTT assay, it was found that MV3-$\beta 3$ invasiveness was half the level of invasiveness of MV3 and MV3-neo, whereas migration through uncoated filters was not affected by $\alpha v\beta 3$ expression (Fig 5). Finally, in an in vivo experimental metastasis assay, no lung colonization was observed for MV3-$\beta 3$ in 3 experiments with 5 mice while 73% of mice inoculated i.v. with MV3 cells and 64% of mice inoculated with MV3-neo cells developed lung colonies (Table 1).

DISCUSSION

Strong expression of $\alpha v\beta 3$ is related to tumorigenicity, invasiveness, and metastatic potential of several melanoma cell lines [18,19,20,21] and $\alpha v\beta 3$ emerges with tumor progression in situ in a large part of the melanoma cases [7,22,23,24]. Furthermore, $\alpha v\beta 3$ ligation was found to facilitate invasion of certain melanoma cells [25], and expression of $\alpha v\beta 3$ has been shown to enhance M21 melanoma tumorigenicity [26] and to provide CS-1 melanoma cells with the capacity to metastasize from the chorioallantoic membrane in a chick embryo [27]. Finally, an antibody to the αv-subunit could block melanoma tumor growth in nude mice [28]. These data suggest that $\alpha v\beta 3$ plays an important role in tumor progression of many melanomas. However, to the best of our knowledge no experimental evidence is available for a direct

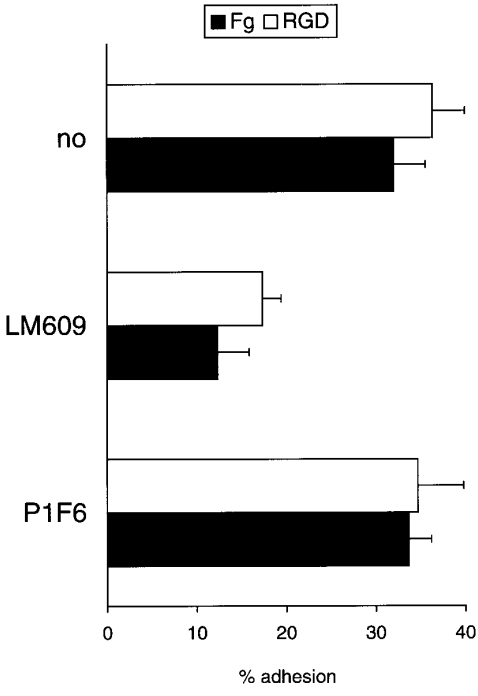

FIG. 3. Inhibition of adhesion of MV3-β3 with anti-αvβ3 mAb. MV3-β3 cells were allowed to adhere to wells coated with 25 μg/ml BSA-GRGDSP or Fg in the absence or presence of LM609 anti-αvβ3 or P1F6 anti-αvβ5 function blocking mAbs. Mean ± s.d. from triplicate determinations is shown for 1 experiment of 3.

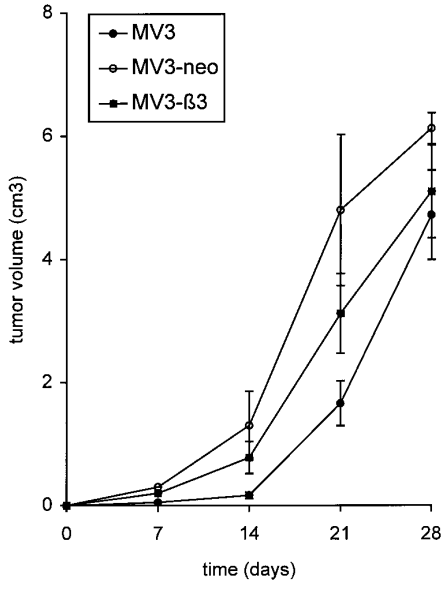

FIG. 4. Effect of αvβ3 on tumorigenicity of MV3 cells. 2×10^5 MV3, MV3-neo, or MV3-β3 cells were s.c. inoculated and tumor volume was measured weekly. Mean ± s.d. from 5 mice is shown for 1 experiment of 2.

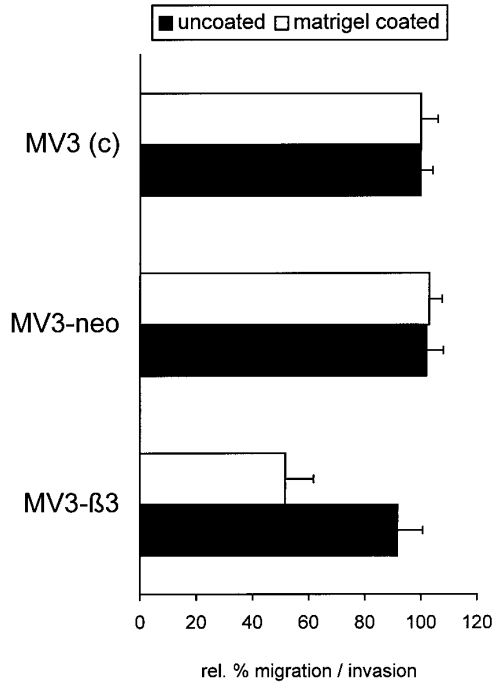

FIG. 5. Effect of $\alpha v\beta 3$ on invasion of MV3 cells. Absorbance of samples from MV3 cells that invaded through matrigel-coated or migrated through uncoated filters was measured. This control value (c) was normalized to 100% and the relative migratory/invasive potential of MV3-neo and MV3-$\beta 3$ cells is shown. Mean ± s.d. of duplicate determinations of 1 of 2 experiments is shown.

causal role for $\alpha v\beta 3$ in melanoma metastasis in mice. Parenthetically, $\alpha v\beta 3$ on endothelial cells is crucial for angiogenesis in melanomas [29], and thus, in a more indirect manner, for melanoma growth.

The fact that MV3 is highly tumorigenic and metastatic while it lacks $\alpha v\beta 3$ [7,13] seems surprising, but it confirms earlier reports of melanoma cell lines that grow and metastasize in the absence of this integrin [6,8]. Together, these reports demonstrate that $\alpha v\beta 3$ may be important for growth, invasion, and metastasis of some melanoma cell lines, but that other melanoma cell lines use alternative mechanisms that do not involve this integrin. Moreover, downmodulation of $\alpha v\beta 3$ expression has been shown to enhance A375m melanoma cell invasiveness in vitro [8], indicating that $\alpha v\beta 3$ can actually inhibit invasion of some cell lines. Our finding that $\alpha v\beta 3$ expression in MV3 reduces its in vitro invasive capacity as well as its ability to form lung colonies upon i.v. inoculation into nude mice, extends this finding.

TABLE 1
Experimental Metastasis of MV3, MV3-neo, and MV3-$\beta 3$

Cell line	Exp. 1	Exp. 2	Exp. 3	Total	%	*p*
MV3	3/5[a]	4/5	4/5	11/15	73	
MV3-neo	2/5	3/4	4/5	9/14	64	>0.05
MV3-$\beta 3$	0/5	0/5	0/5	0/15	0	0.0001

[a] Number of mice with lung colonies/number of mice inoculated.

We do not know how $\alpha v\beta 3$ inhibits MV3 invasion and experimental metastasis. As we have no evidence for a role for $\alpha v\beta 5$ in the adhesive phenotype of MV3 ($\alpha v\beta 5$ is not involved in MV3 adhesion to Fn [30] and we could not detect MV3 adhesion to vitronectin (not shown), Fg, or RGD), loss of the low surface expression of $\alpha v\beta 5$ is unlikely to be responsible for the observed changes in behavior of MV3, though we cannot absolutely rule this out.

Firm $\alpha v\beta 3$-mediated adhesion to Fn may overrule $\beta 1$ integrin-mediated interactions with Fn involved in cell migration [31]. Alternatively, $\alpha v\beta 3$ signaling may affect the function of other integrins, and as a result interfere with invasive behavior. Our finding that the acquired adhesion of MV3-$\beta 3$ to RGD and Fg is not completely blocked by the LM609 anti-$\alpha v\beta 3$ mAb, may indeed suggest that other integrins (that do not mediate binding of the parental cell line to RGD and Fg), have become activated in MV3-$\beta 3$. In addition, $\alpha v\beta 3$ expression may interfere with the complex of signals derived from other integrins that regulates protease expression and cell proliferation [3,4,32]. For all 3 hypotheses, it is important to realize that in our experiments, $\alpha v\beta 3$ expression is forced onto cells that have adopted a strategy for invasion and metastasis in the absence of this integrin. In such a cellular background (integrin/protease profile, etc.) $\alpha v\beta 3$ may be obstructive while it may be part of the metastatic design in other melanoma cells.

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