**αv-INTEGRINS IN HUMAN MELANOMA: GAIN OF αvβ3 AND LOSS OF αvβ5 ARE RELATED TO TUMOR PROGRESSION IN SITU BUT NOT TO METASTATIC CAPACITY OF CELL LINES IN NUDE MICE**

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We investigated the expression of αv-integrins in different stages of human cutaneous melanocytic tumor progression. We observed that αvβ5 was the αv-integrin expressed in all common nevocellular nevi, in 78% of dysplastic nevi, in 63% of early primary melanomas, in 43% of advanced primary melanomas, and in 33% of melanoma metastases. Hence, loss of αvβ5 expression was related to melanocytic tumor progression. In line with earlier reports, αvβ3 was exclusively detected in advanced primary melanomas and metastases (24% and 50% respectively). Staining with anti-αv monoclonal antibodies (MAbs) in lesions where both αvβ3 and αvβ5 were absent showed that alternative αv-integrins were expressed in advanced primary melanomas and metastases. By FACS analysis, we determined expression of αvβ3 and αvβ5 in 4 human melanoma cell lines with different metastatic capacities after s.c. inoculation into nude mice. One of the non-metastatic and both highly metastatic cell lines expressed αvβ3 at their surface. Surprisingly, αvβ3 was detected exclusively in the non-metastatic cell lines. Absence of αvβ5 in the highly metastatic cell lines was confirmed by lack of immunoprecipitation from [35S]-methionine-labeled cells and by absence of immunohistochemical staining on primary and metastatic xenograft lesions. Our findings indicate that cα/Ps expression is often lost in advanced stages of melanocytic tumor progression in situ, while αvβ5 is acquired, but that a decrease in αvβ5 and an increase in αvβ3 expression are not necessarily related to the metastatic behavior of human melanoma cells in nude mice.

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The extracellular matrix (ECM) regulates a number of cellular processes, while integrins link the ECM to structural elements in the cell and play a role as signalling receptors (Hamann, 1992). Therefore, it is likely that integrins can mediate ECM control of cell growth, migration and invasion. These processes play an important role in tumorigenicity and metastasis formation and integrins have indeed been shown to be involved in both occurrences (Juliano and Varner, 1993).

In human melanoma, integrins have been shown to be involved in tumor growth and metastatic spread (Mortarini and Anichini, 1993). For melanocytic tumor progression in situ, changes in the expression of several integrins have been reported, including acquired expression of αvβ3 in the vertical growth phase of primary melanomas and in metastases (Albelda et al., 1990). The αv-subunit of this integrin, however, can be associated with several different β-subunits in melanoma cells in vitro (Marshall et al., 1991) and is expressed in all stages of melanocytic tumor progression in situ (Danen et al., 1994). In this study we have investigated expression of αv-integrins in cutaneous melanocytic lesions and in a panel of human melanoma cell lines with different metastatic capacities in nude mice.

**MATERIAL AND METHODS**

**Lesions**

Specimens were obtained from patients at the University Hospital, Nijmegen, The Netherlands and at the University Hospital, Würzburg, Germany. Based on histopathologic examination of paraffin sections, lesions were divided into 5 classes: common nevocellular nevus (NN) (n = 19), dysplastic (atypical) nevus (DN; De Wit et al., 1993) (n = 9), early primary melanoma (tumor thickness ≤ 1.5 mm; ePM) (n = 8), advanced primary melanoma (tumor thickness > 1.5 mm; aPM) (n = 21) and melanoma metastasis (MM) (n = 24). Representative samples were snap-frozen in liquid nitrogen and stored at −80°C until sectioning.

**Cell lines and culture conditions**

The melanoma cell lines used included: IF6 (Van Muijen et al., 1991a), 530 (Versteeg et al., 1988), BLM (Van Muijen et al., 1991a), MV3 (Van Muijen et al., 1991b) and MelS7 (Van Muijen et al., 1991a). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Flow, Irvine, UK) supplemented with 10% FCS, penicillin and streptomycin.

**Monoclonal antibodies**

The anti-integrin MAbs used were: 4B4 anti-b1 (Morimoto et al., 1985) (Coulter, Hialeah, FL), LM142 anti-αv and LM609 anti-αvβ3 (Cheresh and Spiro, 1987) (kind gifts from Dr. D. Cheresh, La Jolla, CA), 13C2 anti-αv and 23C6 anti-αvβ3 (Davies et al., 1989) (kind gifts from Dr. M. Horton, London, UK), and P1F6 anti-αvβ5 (Wayner et al., 1991) (Telios, San Diego, CA). In FACS analyses WT31 anti-CD3 MAbS (Tax et al., 1983) (kind gift from Dr. W. Tax, Nijmegen, The Netherlands) were used as a negative control. NKK-betab anti- gp100 MAbs (Adema et al., 1993) (kind gift from Dr. C. Figdor, Nijmegen, The Netherlands) were used to identify melanocytic cells in human lesions.

**Immunohistochemistry**

Identical procedures were used for immunohistochemistry of frozen sections of human melanocytic lesions and melanoma cell line xenograft lesions. Frozen sections of 4 μm were fixed in acetone for 10 min and incubated at room temperature with MAbs for 1 hr. After washing with PBS, bound MAbs were visualized by means of the peroxidase-based Vectastain elite ABC kit (Vector, Burlingame, CA) with 3-amino-9-ethylcarbazole as substrate. After counterstaining with Mayer's hematoxylin, sections were mounted with Kaiser's glycerin/gelatin (Merck, Darmstadt, Germany).

Melanocytic cells were identified in H. and E.-stained sections and by staining with NKK-betab MAbs. The percentage of stained melanocytic cells was estimated as 0, 1–25%, 26–50%, 51–75%, or 76–100%. Slides were read independently by 2 observers. Discrepancies exceeding more than one percentage class were found in less than 10% of the cases. These cases were re-evaluated jointly until consensus was reached.

Logistic regression was used to determine a correlation between antigen expression and tumor progression.

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Flow cytometry

Cells were harvested by short trypsinization of subconfluent monolayers and suspended in DMEM/10% FCS. After washing with PBS containing 0.5% BSA and 0.02% azide, they were incubated with MAbs in PBS/BSA/azide for 30 min at 4°C. After washing with PBS/BSA/azide, the cells were incubated with fluorescein-isothiocyanate (FITC)-conjugated F(ab')2 fragments of rabbit anti-mouse Ig antibodies (Dako, Glostrup, Denmark). Analyses were performed with an Epics Elite flow cytometer (Coulter, Mijdrecht, The Netherlands).

Immunoprecipitation

Immunoprecipitations were performed as described earlier (Danen et al., 1993). Briefly, cells were labeled overnight with 0.3 mCi 35S-methionine (Amersham, Houten, The Netherlands), washed and lysed with 0.5% NP40 lysis buffer. Glycoproteins were isolated from NP40-solubilized cell extracts by adsorption to concanavalin A (Con A) Sepharose (Pharmacia, Uppsala, Sweden). To compare the amount of glycoproteins in the different cell lines, equal numbers of counts of the Con A-bound fractions were used for immunoprecipitation. MAbs, rabbit anti-mouse Ig (Dako, Glostrup, Denmark) and ProtA beads (Pharmacia) were subsequently added and the volume was adjusted to 1 ml with 0.5% NP40. Samples were tumbled overnight at 4°C and the beads washed 3 times with 0.5% NP40 and 5 times with 0.5% NP40/0.1% SDS. Finally, the beads were resuspended in sample buffer containing 2-mercaptoethanol, then boiled and run on SDS-PAGE.

RESULTS

Expression of $\alpha_v\beta_3$ in situ

In order to investigate which $\alpha_v$-integrins are expressed in benign melanocytic lesions and to see whether other $\alpha_v$-integrins besides $\alpha_v\beta_3$ are expressed in malignant melanoma, we stained a series of NN, DN, ePM, aPM and MM with P1F6 anti-$\alpha_v\beta_3$ MAbs. Besides staining of fibroblast-like cells in stroma of all lesions, staining of melanocytic cells was found in 100% of NN (19/19), in 78% of DN (7/9), in 63% of ePM (5/8), in 43% of aPM (9/21) and in 33% of MM (8/24) (Figs. 1, 2). Hence, loss of expression of $\alpha_v\beta_3$ was related to tumor progression (CHI$^2$ trend 1 d.f. = 16.3; $p = 0.0001$). In the lesions that were positive for $\alpha_v\beta_3$, a variable heterogeneous staining pattern was observed with 25–100% positive melanocytic cells.

Expression of other $\alpha_v$-integrins in situ

In contrast to $\alpha_v\beta_3$, staining for $\alpha_v\beta_3$ was absent in NN, DN and ePM, whereas 24% of aPM (5/21) and 50% of MM (12/24) were positive, indicating that $\alpha_v\beta_3$ emerged in aPM and MM (CHI$^2$ trend 1 d.f. = 14.4; $p = 0.0001$) (Figs. 1, 2). Incubation with 23C6 or LM609 anti-avp3 MAbs resulted in staining of all the different cell lines, equal numbers of counts of the positive lesions. In all lesions, staining of blood vessels was observed. In 2 DN, 4 ePM, 10 aPM and 6 MM neither $\alpha_v\beta_3$ nor $\alpha_v\beta_3$ could be detected and we incubated these lesions with 13C2 and LM609 anti-avp3 MAbs. Staining was negative for the 2 DN, 4 aPM and 6 MM lesions, whereas 5/10 aPM and 5/6 MM lesions were positive, indicating that other $\alpha_v$-integrins were expressed (not shown). Incubation with 4B4 anti-avp3 MAbs resulted in staining of all melanocytic cells in all lesions (not shown), indicating that $\alpha_v\beta_3$ may possibly be the $\alpha_v$-integrin expressed in aPM and MM lesions.

Expression of $\alpha_v\beta_3$ and $\alpha_v\beta_3$ in human melanoma cell lines

We next examined whether decreased expression of $\alpha_v\beta_3$ and increased expression of $\alpha_v\beta_3$ also correlated with the metastatic potential of cultured human melanoma cell lines. For this purpose we used a panel of 4 human melanoma cell lines. After s.c. inoculation into nude mice, all 4 cell lines were seen to be tumorigenic but IF6 and 530 gave rise to metastases in only a very low percentage of mice or in none at all, whereas BLM and MV3 very frequently metastasized (Van Muijen et al., 1991a, b). FACS analysis showed that comparable levels of $\alpha_v\beta_3$ were expressed on IF6, BLM and MV3 whereas no $\alpha_v\beta_3$ could be detected on 530 cells (Fig. 3a). Surprisingly, no $\alpha_v\beta_3$ was detected on the highly metastatic cell lines BLM and MV3, whereas the non-metastatic cell lines IF6 and 530 expressed $\alpha_v\beta_3$ at their surface (Fig. 3b). Hence, the relation of decreased $\alpha_v\beta_3$ and increased $\alpha_v\beta_3$ expression with melanocytic tumor progression in situ was not paralleled by a relation with the metastatic capacity of human melanoma cells in nude mice.

Biosynthesis of $\alpha_v\beta_3$ in human melanoma cell lines

In order to investigate whether the absence of $\alpha_v\beta_3$ from the surface of BLM and MV3 cells was reflected by a lack of biosynthesis of $\alpha_v\beta_3$ in these cells, immunoprecipitations were performed on 35S-methionine-labeled cells. Consistent with the surface expression data, synthesis of $\alpha_v\beta_3$ was extremely low for the highly metastatic BLM and MV3 cells. For the non-metastatic IF6 and 530 cells, a clear 125-kDa band corresponding to $\alpha_v$ and a 105-kDa band corresponding to $\beta_3$ were detected, whereas these bands were barely visible for BLM and MV3 cells (Fig. 4). The 90-kDa and 150-kDa bands were non-specific since they could be detected even after incubation with normal mouse serum (NMS).

Expression of $\alpha_v\beta_3$ in xenograft lesions

In order to exclude the possibility that absence of $\alpha_v\beta_3$ in the highly metastatic cell lines in vitro was due to culture conditions, we stained xenograft lesions of these cell lines with anti-$\alpha_v\beta_3$ MAbs. No $\alpha_v\beta_3$ was detected in primary tumors or metastases of BLM and MV3 cells, whereas control anti-$\beta_3$ MAbs stained all tumor cells (Fig. 5). For IF6 and 530 cells that expressed $\alpha_v\beta_3$ in vitro, we could not detect $\alpha_v\beta_3$ in xenograft lesions (not shown), suggesting that the level of expression was too low for immunohistochemical detection or, alternatively, that culturing the cells might influence the expression of $\alpha_v\beta_3$. Therefore, we stained s.c. xenograft lesions of Mel57 melanoma cells that strongly express $\alpha_v\beta_3$ in vitro (our unpublished data), as a positive control. As shown in Figure 5,
**Figure 2** - Microphotographs of melanocytic lesions stained with anti-\( \alpha \)-integrin MAbs. Melanocytic lesions were stained with 23C6 and LM609 anti-\( \alpha_6 \beta_3 \) or P1F6 anti-\( \alpha_6 \beta_5 \) MAbs. Arrowheads indicate nevus (a-d) or melanoma (e-j) cells. (a, b) NN negative for \( \alpha_6 \beta_3 \) (a) and positive for \( \alpha_6 \beta_5 \) (b). (c, d) DN negative for \( \alpha_6 \beta_3 \) (c; arrow = positive blood-vessel) and positive for \( \alpha_6 \beta_5 \) (d). (e, f) ePM negative for \( \alpha_6 \beta_3 \) (e; arrow = positive blood vessel) and positive for \( \alpha_6 \beta_5 \) (f). (g, h) aPM positive for \( \alpha_6 \beta_3 \) (g) and negative for \( \alpha_6 \beta_5 \) (h) (s = stromal cells). (i, j) MM positive for \( \alpha_6 \beta_3 \) (i) and negative for \( \alpha_6 \beta_5 \) (j) (s = stromal cells). Bars: 20 \( \mu \)m.
with control anti-CD3 MAbs. Results with 23C6 MAbs were identical to those with LM609.

Figure 3 – Surface expression of α-integrins on human melanoma cell lines. IF6 (thin line), 530 (thin dotted line), BLM (thick line) and MV3 (thick dotted line) were incubated with P1F6 anti-αβ3 (a) or LM609 anti-αβ3 MAbs (b) followed by FITC-labeled second antibodies. Fluorescence was measured on an Epics Elite flow cytometer. The vertical line indicates the gate set for the melanoma cell lines, IF6 (thin line), 530 (thin dotted line), BLM (thick line) and MV3 (thick dotted line) were incubated with P1F6 anti-αβ3 MAbs. Results with 23C6 MAbs were identical to those with LM609.

Mel57 melanoma cells stained strongly with LM609 MAbs in xenograft lesions. Hence, in line with the in vitro findings, primary tumors and lung metastases in nude mice of the highly metastatic human melanoma cell lines BLM and MV3 did not show αβ3 expression.

DISCUSSION

Cutaneous melanoma is characterized by proliferative and invasive growth in the dermis and is often followed by widespread metastasis. Interactions of tumor cells with the ECM, which are mainly mediated by integrins (Hynes, 1992), are thought to play an important role in the malignant behavior of melanoma (Mortarini and Anichini, 1993) and other human tumors (Juliano and Varner, 1993). In the present study, we investigated the expression of α-integrins in human melanocytic tumor progression in situ and in a panel of human melanoma cell lines with different metastatic capacities after s.c. inoculation into nude mice.

Acquired expression of αβ3 in the vertical-growth phase of primary melanomas and in melanoma metastases has been reported (Albelda et al., 1990) whereas the α5-subunit is expressed in all stages of melanocytic tumor progression (Danen et al., 1994). In vitro, melanoma cells have been shown to express αβ3, αβ1 (Marshall et al., 1991) and αβ5 (Wayner et al., 1991). In this study we have investigated which of the α-integrins are expressed in situ in nevi and in primary melanomas and metastases. Our data concerning αβ3 expression confirm the findings from previous studies (Albelda et al., 1990) and show that αβ3 emerges in aPM and MM. Regarding αβ3, we find that expression is often lost with melanocytic tumor progression. Since no MAbs have yet been generated recognizing the αβ3 complex, we could not investigate expression of this integrin. For the lesions with αβ3 and/or αβ3 expression we cannot exclude the possibility that other α-integrins are expressed as well. For those lesions in which neither αβ3 nor αβ3 was detected, we investigated whether alternative α-integrins were expressed. The fact that alternative α-integrins were not detected in DN or ePM, whereas a number of PM and MM did express other α-integrins, suggests that additional α-integrins may emerge in aPM and MM besides αβ3. Thus, in NN, DN and ePM, αβ3 may be expressed, whereas in aPM and MM αβ5, αβ3 and/or other α-integrins may be expressed. Since all lesions were β1-positive, and since αβ1 can be expressed by melanoma cells in vitro (Marshall et al., 1991), αβ1 may be the alternative emerging α-integrin.

The fact that αβ3 expression is lost in most MM and that expression of αβ3 emerges may have functional consequences for the melanoma cells. The ligand-binding specificity of αβ5 seems to be restricted to vitronectin whereas αβ3 recognizes multiple ligands including vitronectin and fibronectin (Smith et al., 1990). In addition, αβ3 and αβ3 promote distinct cellular responses to vitronectin in vitro (Leavesey et al., 1992). We have recently shown that, for melanomas originating from the uvea, αβ3 is absent in all primary lesions including those of the aggressive type, and that αβ3 is the α-integrin expressed (Ten Berge et al., 1993). This may indicate that the microenvironment of the melanoma cells is important in determining which α-integrins are expressed. The fact that a role in proliferation (Pehling-Habermann et al., 1992) and invasion (Setfor et al., 1992) of melanoma cells has been attributed to αβ3 seems to be in line with the emergence of αβ3 in aPM and MM. In our panel of human melanoma cell lines, IF6 and 530 express αβ3 in vitro but no αβ3 can be detected in the primary xenograft.

Figure 4 – Biosynthesis of αβ3 in human melanoma cell lines. IF6, 530, BLM and MV3 were metabolically labeled with 35S-methionine and lysed, then glycoproteins were isolated on Con A Sepharose. Equal numbers of Con A-bound counts were used for immunoprecipitation with 23C6 anti-αβ3 MAbs or NMS as a negative control. Identical results were obtained with LM609 MAbs.
tumors, which grow slowly. On the other hand, BLM and MV3 tumors lack $\alpha_6\beta_3$ and grow rapidly. All 4 cell lines develop tumors upon s.c. inoculation into nude mice (Van Muijen et al., 1991a, b). This suggests that $\alpha_6\beta_3$ is not necessarily involved in melanoma tumor growth. Furthermore, the fact that, for the highly metastatic cell lines, $\alpha_6\beta_3$ expression is absent in vitro, in s.c. xenograft lesions and in lung metastases, suggests that they use alternative integrins for metastasizing in nude mice.

In conclusion, we show that $\alpha_6\beta_5$ is often lost in advanced stages of melanocytic tumor progression in situ while $\alpha_6\beta_3$ emerges, but that decrease in $\alpha_6\beta_5$ and increase in $\alpha_6\beta_3$ are not necessarily related to the metastatic potential of human melanoma cell lines in nude mice.

ACKNOWLEDGEMENTS

We thank Drs. D. Cheresh, C. Figdor, M. Horton and W. Tax for generously providing antibodies and Dr. E.-B. Bröcker for kindly providing some of the specimens. We are indebted to Dr. B. van ‘t Hof-Grootenboer for statistical analyses. This work was supported by grant NUKC 91-09 from the Dutch Cancer Society.
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