αv-INTERGINS 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 αvβ3 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 αvβ3 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) directed against αv-integrins was observed that was the Ov-integrin expressed in all common nevocellular nevi (NN) (n = 19), dysplastic (atypical) nevi (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 Mel57 (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-αβ3 and 23C6 anti-αvβ3 (Davies et al., 1989) (kind gifts from Dr. M. Horton, London, UK), and P1F6 anti-αvβ3 (Wayner et al., 1991) (Telios, San Diego, CA). In FACS analyses WT31 anti-CD3 MAb (Tax et al., 1983) (kind gift from Dr. W. Tax, Nijmegen, The Netherlands) were used as a negative control. NKI-beteb 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.

Immunochemistry

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-amin-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 melanosome-stained sections and by staining with NKI-beteb 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 $\alpha_5\beta_3$ in situ

In order to investigate which $\alpha_5$-integrins are expressed in benign melanocytic lesions and to see whether other $\alpha_5$-integrins besides $\alpha_5\beta_3$ are expressed in malignant melanoma, we stained a series of NN, DN, ePM, aPM and MM with P1F6 anti-$\alpha_5\beta_3$ 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 $\alpha_5\beta_3$ was related to tumor progression (CHI2 trend 1 d.f. = 16.3; $p = 0.0001$). In the lesions that were positive for $\alpha_5\beta_3$, a variable heterogeneous staining pattern was observed with 25–100% positive melanocytic cells.

Expression of other $\alpha_5$-integrins in situ

In contrast to $\alpha_5\beta_3$, staining for $\alpha_5\beta_1$ was absent in NN, DN and ePM, whereas 24% of aPM (5/21) and 50% of MM (12/24) were positive, indicating that $\alpha_5\beta_1$ emerged in aPM and MM (CHI2 trend 1 d.f. = 14.4; $p = 0.0001$) (Figs. 1, 2). Incubation with 23C6 or LM609 anti-$\alpha_5\beta_1$ MAbs gave similar results. In all lesions, staining of blood vessels was observed. In 2 DN, 4 ePM, 10 aPM and 6 MM neither $\alpha_5\beta_3$ nor $\alpha_5\beta_1$ could be detected and we incubated these lesions with 13C2 and LM609 anti-$\alpha_5\beta_3$ MAbs. Staining was negative for the 2 DN and 4 ePM lesions, whereas 5/10 aPM and 5/6 MM lesions were positive, indicating that other $\alpha_5$-integrins were expressed (not shown). Incubation with 4B4 anti-$\alpha_1$ MAbs resulted in staining of all melanocytic cells in all lesions (not shown), indicating that $\alpha_5\beta_1$ may possibly be the $\alpha_5$-integrin expressed in aPM and MM lesions.

Expression of $\alpha_5\beta_3$ and $\alpha_5\beta_3$ in human melanoma cell lines

We next examined whether decreased expression of $\alpha_5\beta_3$ and increased expression of $\alpha_5\beta_3$ also correlated with the metastatic potential of cultured human melanoma cells. 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 $\alpha_5\beta_3$ were expressed on IF6, BLM and MV3 whereas no $\alpha_5\beta_3$ could be detected on 530 cells. (Fig. 3a). Surprisingly, no $\alpha_5\beta_3$ was detected on the highly metastatic cell lines BLM and MV3, whereas the non-metastatic cell lines IF6 and 530 expressed $\alpha_5\beta_3$ at their surface (Fig. 3b). Hence, the relation of decreased $\alpha_5\beta_3$ and increased $\alpha_5\beta_3$ 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 $\alpha_5\beta_3$ in human melanoma cell lines

In order to investigate whether the absence of $\alpha_5\beta_3$ from the surface of BLM and MV3 cells was reflected by a lack of biosynthesis of $\alpha_5\beta_3$ in these cells, immunoprecipitations were performed on 35S-methionine-labeled cells. Consistent with the surface expression data, synthesis of $\alpha_5\beta_3$ 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 $\alpha_5$ 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 $\alpha_5\beta_3$ in xenograft lesions

In order to exclude the possibility that absence of $\alpha_5\beta_3$ in the highly metastatic cell lines in vitro was due to culture conditions, we stained xenograft lesions of these cell lines with anti-$\alpha_5\beta_3$ MAbs. No $\alpha_5\beta_3$ 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 $\alpha_5\beta_3$ in vitro, we could not detect $\alpha_5\beta_3$ 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 $\alpha_5\beta_3$. Therefore, we stained s.c. xenograft lesions of Mel57 melanoma cells that strongly express $\alpha_5\beta_3$ in vitro (our unpublished data), as a positive control. As shown in Figure 5,

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Expression of $\alpha_5\beta_3$ and $\alpha_5\beta_3$ in different stages of human melanocytic tumor progression. Nineteen NN, 9 DN, 8 ePM, 21 aPM and 24 MM were stained with P1F6 anti-$\alpha_5\beta_3$ and 23C6 and LM609 anti-$\alpha_5\beta_3$ MAbs. Percentages of lesions in which positive melanocytic cells were observed are indicated. In the lesions that were positive, 25–100% of melanocytic cells stained.
\end{figure}
Figure 2 - Microphotographs of melanocytic lesions stained with anti-αv-integrin MAbs. Melanocytic lesions were stained with 23C6 and LM609 anti-αvβ3 or P1F6 anti-αvβ5 MAbs. Arrowheads indicate nevus (a–d) or melanoma (e–j) cells. (a, b) NN negative for αvβ3 (a) and positive for αvβ5 (b). (c, d) DN negative for αvβ3 (c; arrow = positive blood-vessel) and positive for αvβ5 (d). (e, f) ePM negative for αvβ3 (e; arrow = positive blood-vessel) and positive for αvβ5 (f). (g, h) aPM positive for αvβ3 (g) and negative for αvβ5 (h) (s = stromal cells). (i, j) MM positive for αvβ3 (i) and negative for αvβ5 (j) (s = stromal cells). Bars: 20 μm.
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 (Hyne, 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 αv-subunit is expressed in all stages of melanocytic tumor progression (Danen et al., 1994). In vitro, melanoma cells have been shown to express αvβ3, αvβ1 (Marshall et al., 1991) and αvβ5 (Wayner et al., 1991). In this study, we have investigated which of the αv-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 αβ6, we find that expression is often lost with melanocytic tumor progression. Since no MAbs have yet been generated recognizing the αβ complex, we could not investigate expression of this integrin. For the lesions with αvβ3 and/or αvβ5 expression, we cannot exclude the possibility that other αv-integrins are expressed as well. For those lesions in which neither αvβ3 nor αvβ5 was detected, we investigated whether alternative αv-integrins were expressed. The fact that alternative αv-integrins were not detected in DN or cPM, whereas a number of PM and MM did express other αv-integrins, suggests that additional αv-integrins may emerge in aPM and MM besides αvβ3. Thus, in NN, DN, and cPM, αvβ3 may be expressed, whereas in aPM and MM αvβ3, αvβ5, and/or other αv-integrins may be expressed. Since all lesions were αv-negative, and since αvβ1 can be expressed by melanoma cells in vitro (Marshall et al., 1991), αvβ1 may be the emerging αv-integrin.

The fact that αvβ3 expression is lost in most MM and that expression of αvβ3 emerges may have functional consequences for the melanoma cells. The ligand-binding specificity of αvβ3 seems to be restricted to vitronectin whereas αvβ1 recognizes multiple ligands including vitronectin and fibronectin (Smith et al., 1990). In addition, αvβ3 and αvβ5 promote distinct cellular responses to vitronectin in vitro (Leavesey et al., 1992). We have recently shown that, for melanomas originating from the uvea, αvβ3 is absent in all primary lesions including those of the aggressive type, and that αvβ3 is the αv-integrin expressed (Ten Barge et al., 1993). This may indicate that the microenvironment of the melanoma cells is important in determining which αv-integrins are expressed. The fact that a role in proliferation (Felding-Habermann et al., 1992) and invasion (Settor et al., 1992) of melanoma cells has been attributed to αvβ3 seems to be in line with the emergence of αvβ3 in aPM and MM. In our panel of human melanoma cell lines, IF6 and 530 express αvβ3 in vitro but no αvβ3 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 $\alpha_v\beta_3$ 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 $\alpha_v\beta_3$ is not necessarily involved in melanoma tumor growth. Furthermore, the fact that, for the highly metastatic cell lines, $\alpha_v\beta_3$ 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 $\alpha_v\beta_5$ is often lost in advanced stages of melanocytic tumor progression in situ while $\alpha_v\beta_3$ emerges, but that decrease in $\alpha_v\beta_5$ and increase in $\alpha_v\beta_3$ are not necessarily related to the metastatic potential of human melanoma cell lines in nude mice.

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