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α_v-INTEGRINS IN HUMAN MELANOMA: GAIN OF $\alpha_v\beta_3$ AND LOSS OF $\alpha_v\beta_5$ 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 α_v-integrins in different stages of human cutaneous melanocytic tumor progression. We observed that $\alpha_v\beta_5$ was the α_v-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 $\alpha_v\beta_5$ expression was related to melanocytic tumor progression. In line with earlier reports, $\alpha_v\beta_3$ was exclusively detected in advanced primary melanomas and metastases (24% and 50% respectively). Staining with anti-α_v monoclonal antibodies (MAbs) in lesions where both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were absent showed that alternative α_v-integrins were expressed in advanced primary melanomas and metastases. By FACS analysis, we determined expression of $\alpha_v\beta_5$ and $\alpha_v\beta_3$ 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 $\alpha_v\beta_5$ at their surface. Surprisingly, $\alpha_v\beta_3$ was detected exclusively in the non-metastatic cell lines. Absence of $\alpha_v\beta_3$ in the highly metastatic cell lines was confirmed by lack of immunoprecipitation from ^{35}S-methionine-labeled cells and by absence of immunohistochemical staining on primary and metastatic xenograft lesions. Our findings indicate that $\alpha_v\beta_5$ expression is often lost in advanced stages of melanocytic tumor progression *in situ*, while $\alpha_v\beta_3$ is acquired, but that a decrease in $\alpha_v\beta_5$ and an increase in $\alpha_v\beta_3$ expression are not necessarily related to the metastatic behavior of human melanoma cells in nude mice.

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 (Hynes, 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 $\alpha_v\beta_3$ in the vertical growth phase of primary melanomas and in metastases (Albelda *et al.,* 1990). The α_v-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 α_v-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.,* 1991*a*), 530 (Versteeg *et al.,* 1988), BLM (Van Muijen *et al.,* 1991*a*), MV3 (Van Muijen *et al.,* 1991*b*) and Mel57 (Van Muijen *et al.,* 1991*a*). 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-β1 (Morimoto *et al.,* 1985) (Coulter, Hialeah, FL), LM142 anti-α_v and LM609 anti-$\alpha_v\beta_3$ (Cheresh and Spiro, 1987) (kind gifts from Dr. D. Cheresh, La Jolla, CA), 13C2 anti-α_v and 23C6 anti-$\alpha_v\beta_3$ (Davies *et al.,* 1989) (kind gifts from Dr. M. Horton, London, UK), and P1F6 anti-$\alpha_v\beta_5$ (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. 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.

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 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 ^{35}S-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 $\alpha_v\beta_5$ in situ

In order to investigate which α_v-integrins are expressed in benign melanocytic lesions and to see whether other α_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-$\alpha_v\beta_5$ 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 $\alpha_v\beta_5$ was related to tumor progression (CHI^2 trend 1 d.f. = 16.3; $p = 0.0001$). In the lesions that were positive for $\alpha_v\beta_5$, a variable heterogeneous staining pattern was observed with 25–100% positive melanocytic cells.

Expression of other α_v-integrins in situ

In contrast to $\alpha_v\beta_5$, 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-$\alpha_v\beta_3$ MAbs gave similar results. In all lesions, staining of blood vessels was observed. In 2 DN, 4 ePM, 10 aPM and 6 MM neither $\alpha_v\beta_3$ nor $\alpha_v\beta_5$ could be detected and we incubated these lesions with 13C2 and LM142 anti-α_v 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 α_v-integrins were expressed (not shown). Incubation with 4B4 anti-β_1 MAbs resulted in staining of all melanocytic cells in all lesions (not shown), indicating that $\alpha_v\beta_1$ may possibly be the α_v-integrin expressed in aPM and MM lesions.

Expression of $\alpha_v\beta_5$ and $\alpha_v\beta_3$ in human melanoma cell lines

We next examined whether decreased expression of $\alpha_v\beta_5$ and increased expression of $\alpha_v\beta_3$ 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.*, 1991*a*, *b*). FACS analysis showed that comparable levels of $\alpha_v\beta_5$ were expressed on IF6, BLM and MV3 whereas no $\alpha_v\beta_5$ could be detected on 530 cells. (Fig. 3*a*). 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. 3*b*). Hence, the relation of decreased $\alpha_v\beta_5$ 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.

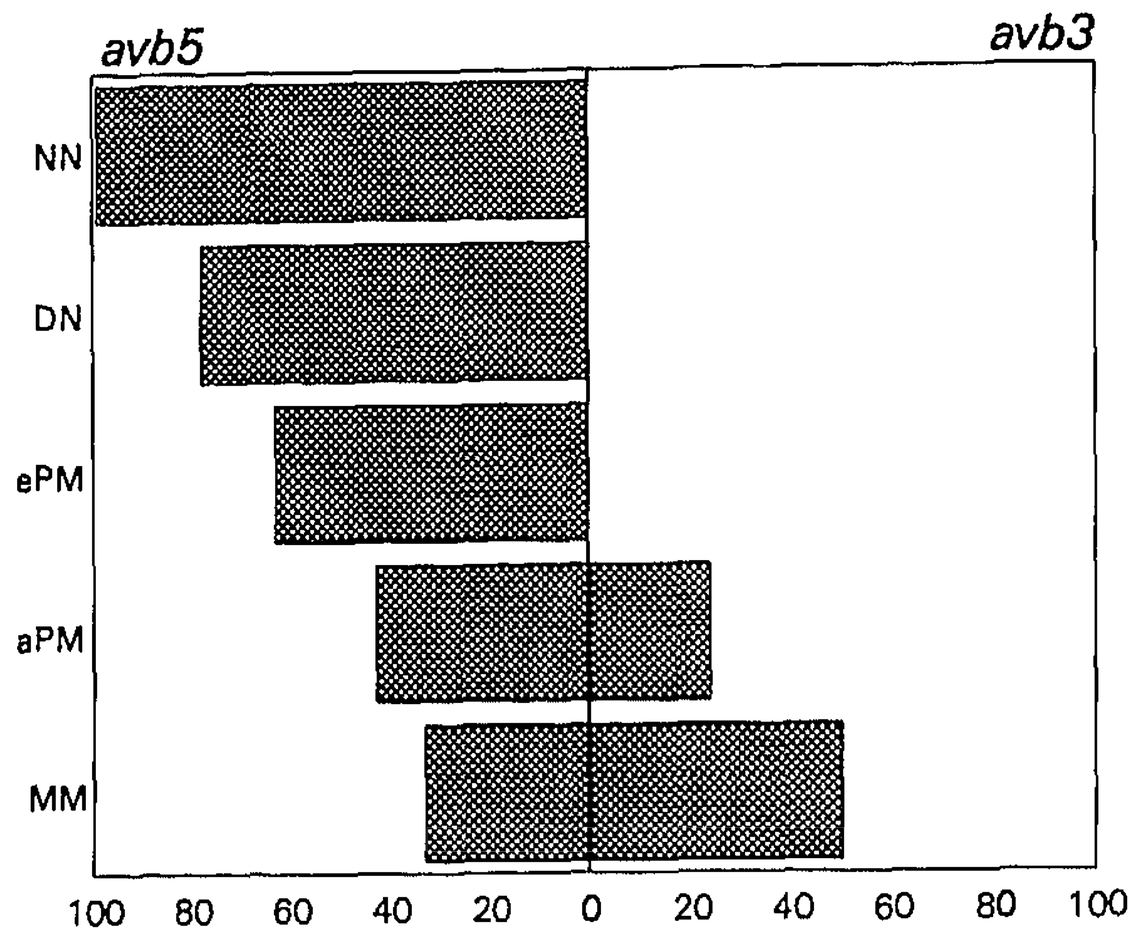

FIGURE 1 – Expression of $\alpha_v\beta_5$ and $\alpha_v\beta_3$ in different stages of human melanocytic tumor progression. Nineteen NN, 9 DN, 8 ePM, 21 aPM and 24 MM were stained with P1F6 anti-$\alpha_v\beta_5$ and 23C6 and LM609 anti-$\alpha_v\beta_3$ MAbs. Percentages of lesions in which positive melanocytic cells were observed are indicated. In the lesions that were positive, 25–100% of melanocytic cells stained.

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 ^{35}S-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 α_v and a 105-kDa band corresponding to β_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-β_1 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,

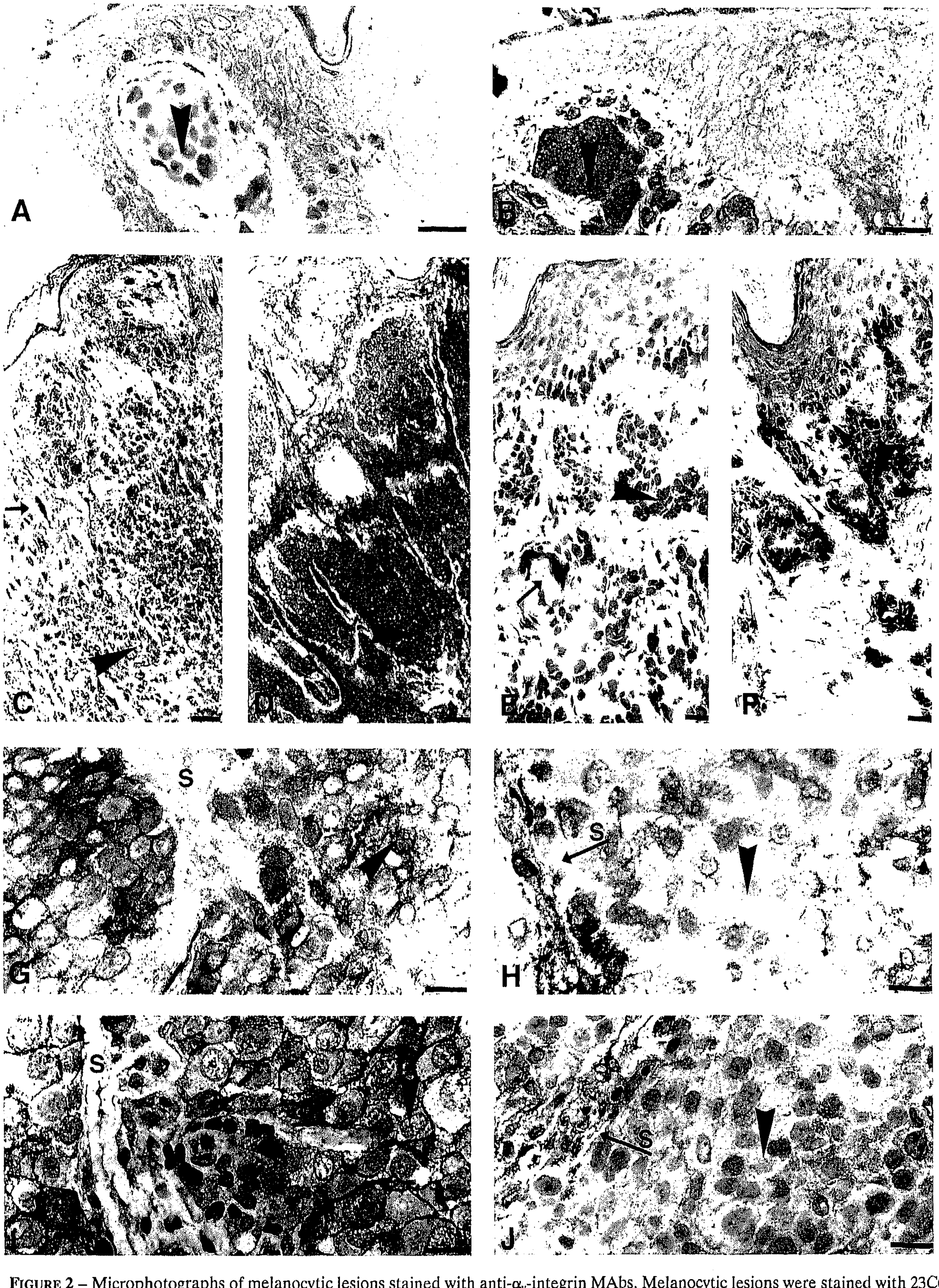

FIGURE 2 – Microphotographs of melanocytic lesions stained with anti-α_v-integrin MAbs. Melanocytic lesions were stained with 23C6 and LM609 anti-$\alpha_v\beta_3$ or P1F6 anti-$\alpha_v\beta_5$ MAbs. Arrowheads indicate nevus *(a–d)* or melanoma *(e–j)* cells. *(a, b)* NN negative for $\alpha_v\beta_3$ *(a)* and positive for $\alpha_v\beta_5$ *(b)*. *(c, d)* DN negative for $\alpha_v\beta_3$ (*c;* arrow = positive blood-vessel) and positive for $\alpha_v\beta_5$ *(d)*. *(e, f)* ePM negative for $\alpha_v\beta_3$ (*e;* arrow = positive blood vessel) and positive for $\alpha_v\beta_5$ *(f)*. *(g, h)* aPM positive for $\alpha_v\beta_3$ *(g)* and negative for $\alpha_v\beta_5$ *(h)* (s = stromal cells). *(i, j)* MM positive for $\alpha_v\beta_3$ *(i)* and negative for $\alpha_v\beta_5$ *(j)* (s = stromal cells). Bars: 20 μm.

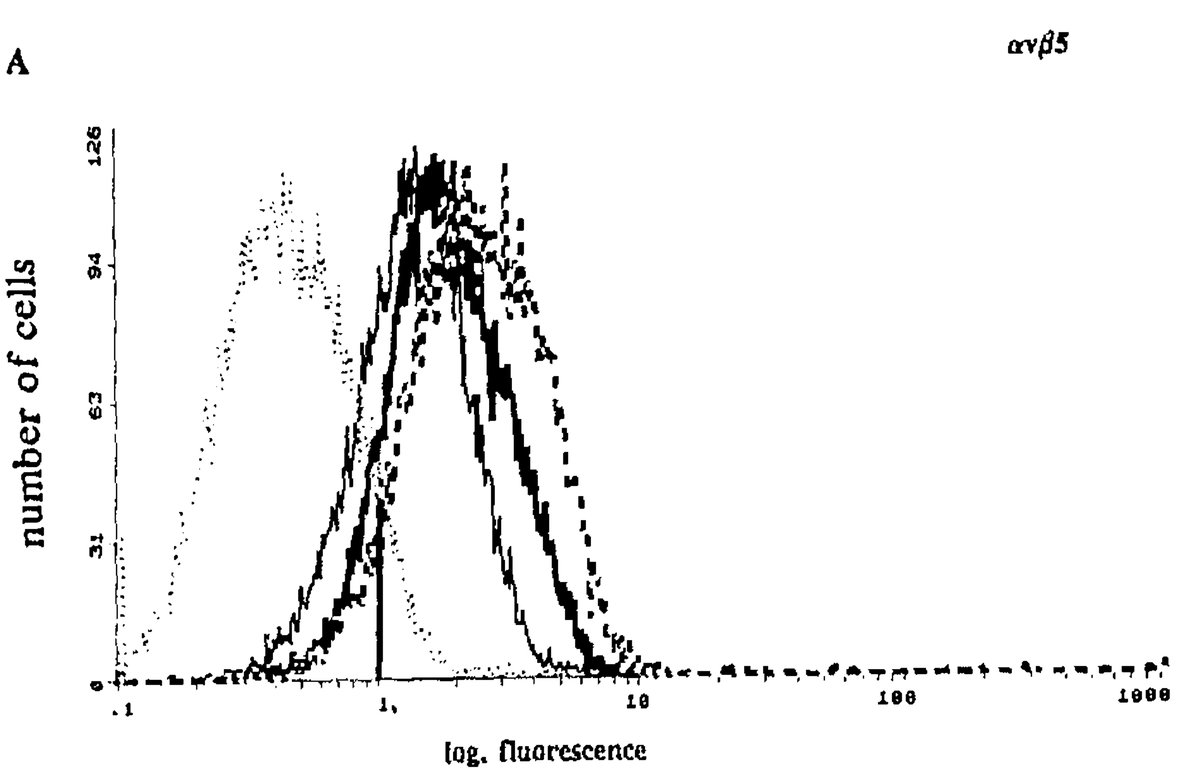

FIGURE 3 – Surface expression of α_v-integrins on human melanoma cell lines. IF6 (thin line), 530 (thin dotted line), BLM (thick line) and MV3 (thick dotted line) were incubated with P1F6 anti-$\alpha_v\beta_5$ *(a)* or LM609 anti-$\alpha_v\beta_3$ MAbs *(b)* followed by FITC-labeled second antibodies. Fluorescence was measured on an Epics Elite flow cytometer. The vertical line indicates the gate set with control anti-CD3 MAbs. Results with 23C6 MAbs were identical to those with LM609.

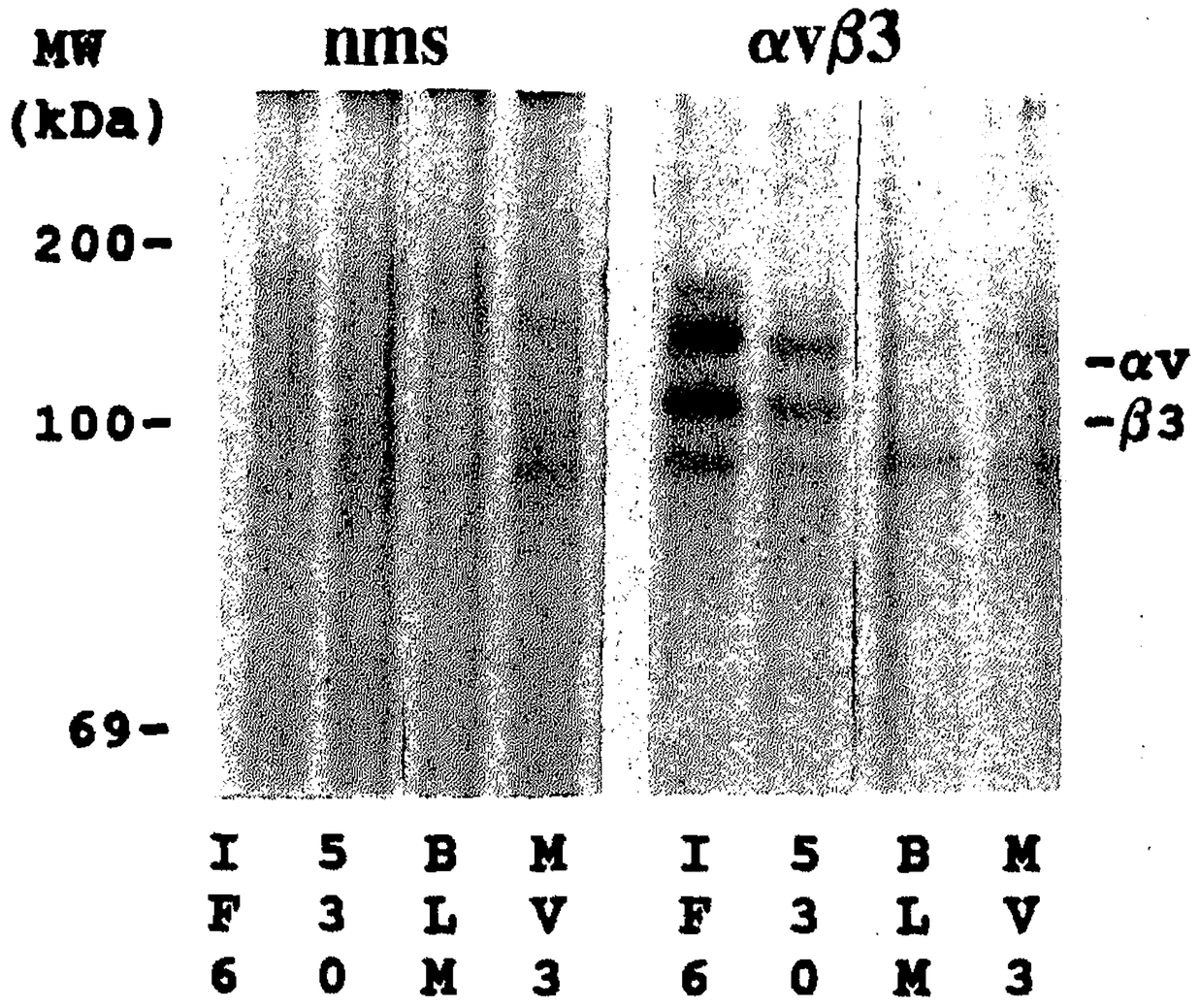

FIGURE 4 – Biosynthesis of $\alpha_v\beta_3$ in human melanoma cell lines. IF6, 530, BLM and MV3 were metabolically labeled with ^{35}S-methionine and lysed, then glycoproteins were isolated on Con A Sepharose. Equal numbers of Con A-bound counts were used for immunoprecipitation with 23C6 anti-$\alpha_v\beta_3$ MAbs or NMS as a negative control. Identical results were obtained with LM609 MAbs.

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 $\alpha_v\beta_3$ 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_v-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 $\alpha_v\beta_3$ in the vertical-growth phase of primary melanomas and in melanoma metastases has been reported (Albelda *et al.,* 1990) whereas the α_v-subunit is expressed in all stages of melanocytic tumor progression (Danen *et al.,* 1994). *In vitro,* melanoma cells have been shown to express $\alpha_v\beta_3$, $\alpha_v\beta_1$ (Marshall *et al.,* 1991) and $\alpha_v\beta_5$ (Wayner *et al.,* 1991). In this study we have investigated which of the α_v-integrins are expressed *in situ* in nevi and in primary melanomas and metastases. Our data concerning $\alpha_v\beta_3$ expression confirm the findings from previous studies (Albelda *et al.,* 1990) and show that $\alpha_v\beta_3$ emerges in aPM and MM. Regarding $\alpha_v\beta_5$, we find that expression is often lost with melanocytic tumor progression. Since no MAbs have yet been generated recognizing the $\alpha_v\beta_1$ complex, we could not investigate expression of this integrin. For the lesions with $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ expression we cannot exclude the possibility that other α_v-integrins are expressed as well. For those lesions in which neither $\alpha_v\beta_3$ nor $\alpha_v\beta_5$ was detected, we investigated whether alternative α_v-integrins were expressed. The fact that alternative α_v-integrins were not detected in DN or ePM, whereas a number of PM and MM did express other α_v-integrins, suggests that additional α_v-integrins may emerge in aPM and MM besides $\alpha_v\beta_3$. Thus, in NN, DN and cPM, $\alpha_v\beta_5$ may be expressed, whereas in aPM and MM $\alpha_v\beta_5$, $\alpha_v\beta_3$ and/or other α_v-integrins may be expressed. Since all lesions were β_1-positive, and since $\alpha_v\beta_1$ can be expressed by melanoma cells *in vitro* (Marshall *et al.,* 1991), $\alpha_v\beta_1$ may be the alternative emerging α_v-integrin.

The fact that $\alpha_v\beta_5$ expression is lost in most MM and that expression of $\alpha_v\beta_3$ emerges may have functional consequences for the melanoma cells. The ligand-binding specificity of $\alpha_v\beta_5$ seems to be restricted to vitronectin whereas $\alpha_v\beta_3$ recognizes multiple ligands including vitronectin and fibronectin (Smith *et al.,* 1990). In addition, $\alpha_v\beta_5$ and $\alpha_v\beta_3$ promote distinct cellular responses to vitronectin *in vitro* (Leavesey *et al.,* 1992). We have recently shown that, for melanomas originating from the uvea, $\alpha_v\beta_3$ is absent in all primary lesions including those of the aggressive type, and that $\alpha_v\beta_5$ is the α_v-integrin expressed (Ten Berge *et al.,* 1993). This may indicate that the microenvironment of the melanoma cells is important in determining which α_v-integrins are expressed. The fact that a role in proliferation (Fehlding-Habermann *et al.,* 1992) and invasion (Seftor *et al.,* 1992) of melanoma cells has been attributed to $\alpha_v\beta_3$ seems to be in line with the emergence of $\alpha_v\beta_3$ in aPM and MM. In our panel of human melanoma cell lines, IF6 and 530 express $\alpha_v\beta_3$ *in vitro* but no $\alpha_v\beta_3$ can be detected in the primary xenograft

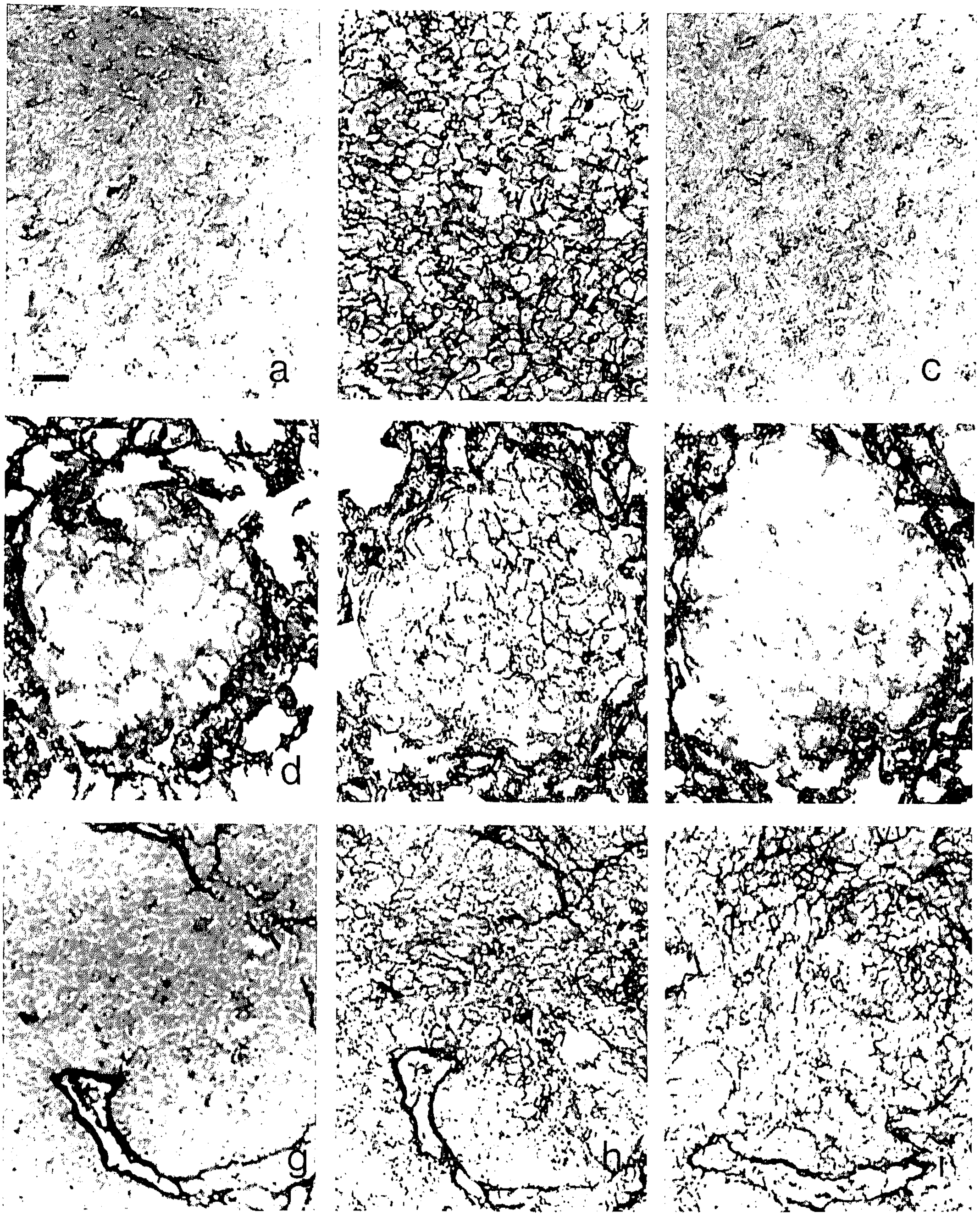

FIGURE 5 – Expression of $\alpha_v\beta_3$ in xenograft lesions. Frozen sections from MV3 s.c. tumor *(a, b, c)*, MV3 lung metastasis *(d, e, f)*, and Mel57 s.c. tumor *(g, h, i)*, were stained either in the absence of primary MAbs *(a, d, g)*, with LM609 anti-$\alpha_v\beta_3$ *(b, e, h)* or with 4B4 anti-β_1 MAbs *(c, f, i)*. Results for BLM were identical to those shown for MV3. Bar: 20 μm.

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.*, 1991*a, 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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