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## B Felding-Habermann, ..., C A Romerdahl, D A Cheresh

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#### Research Article

Human melanoma originates in the skin and can lead to wide-spread metastatic disease. Analysis of melanoma biopsy material has shown that the vitronectin receptor, integrin alpha v beta 3, is a specific marker of the most malignant cells, i.e., vertically invasive primary lesions or distant metastases (Albelda, S. M., S. A. Mette, D. E. Elder, R. Stewart, L. Damjanovich, M. Herlyn, and C. A. Buck. 1990. Cancer Res. 50:6757-6764), suggesting a role for this adhesion receptor in the malignant growth of human melanoma tumors. A cell model was established to analyze the role of alpha v integrins on the tumorigenicity of human melanoma. From M21 human melanoma cells, stable variants were selected that lack alpha v gene expression and thus fail to express integrin alpha v beta 3 (M21-L cells). These cells not only lost the ability to attach to vitronectin but showed a dramatic reduction in tumorigenicity when transplanted into athymic nude mice, compared with M21 cells, even though both cell types showed identical beta 1 integrin expression and growth properties in vitro. M21-L cells were stably transfected with a cDNA-encoding alpha v. This resulted in the functional expression of integrin alpha v beta 3 on these cells and completely restored their tumorigenicity. Thus, integrin alpha v gene expression and the resulting adhesive phenotype are directly involved in the [...]



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### Involvement of Integrin a V Gene Expression in Human Melanoma Tumorigenicity

Brunhilde Felding-Habermann, Barbara M. Mueller, Cynthia A. Romerdahl, and David A. Cheresh Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

#### Abstract

Human melanoma originates in the skin and can lead to widespread metastatic disease. Analysis of melanoma biopsy material has shown that the vitronectin receptor, integrin  $\alpha v\beta 3$ , is a specific marker of the most malignant cells, i.e., vertically invasive primary lesions or distant metastases (Albelda, S. M., S. A. Mette, D. E. Elder, R. Stewart, L. Damjanovich, M. Herlyn, and C. A. Buck. 1990. Cancer Res. 50:6757-6764), suggesting a role for this adhesion receptor in the malignant growth of human melanoma tumors. A cell model was established to analyze the role of  $\alpha v$  integrins on the tumorigenicity of human melanoma. From M21 human melanoma cells, stable variants were selected that lack  $\alpha v$  gene expression and thus fail to express integrin  $\alpha v\beta 3$  (M21-L cells). These cells not only lost the ability to attach to vitronectin but showed a dramatic reduction in tumorigenicity when transplanted into athymic nude mice, compared with M21 cells, even though both cell types showed identical  $\beta$ 1 integrin expression and growth properties in vitro. M21-L cells were stably transfected with a cDNA-encoding  $\alpha v$ . This resulted in the functional expression of integrin  $\alpha v\beta 3$  on these cells and completely restored their tumorigenicity. Thus, integrin  $\alpha v$  gene expression and the resulting adhesive phenotype are directly involved in the proliferation of human melanoma in vivo. (J. Clin. Invest. 1992. 89:2018-2022.) Key words: melanoma proliferation • vitronectin receptor • adhesion

#### Introduction

Integrin-mediated cellular adhesion mechanisms have been proposed to play a key role in various biological properties, including the malignant growth of cancer (1-8). Previous studies indicated a possible role of integrins in tumor formation (3-5) and metastasis (6-8). Specifically, overexpression of the fibronectin receptor  $\alpha 5\beta 1$  suppressed the transformed pheno-

J. Clin. Invest. © The American Society for Clinical Investigation, Inc. 0021-9738/92/06/2018/05 \$2.00 Volume 89, June 1992, 2018–2022 type of Chinese hamster ovary cells (3), and ras-transformed rodent cells showed a reduction in this integrin, compared with their nontransformed counterparts (4).

We have investigated the adhesive and proliferative properties of human melanoma cells. Primary human melanoma proliferates either horizontally or vertically in the skin. It is this vertical growth phase that typically leads to widespread metastatic disease. Albelda et al. (9) examined normal skin melanocytes, nevi, and primary and metastatic melanoma tissues for the expression of a number of integrins. These investigators demonstrated that the vitronectin receptor, integrin  $\alpha v\beta 3$ , was preferentially detected on vertical growth phase primary melanoma as well as metastatic lesions, and was not detected on melanocytes, nevi, or horizontal primary melanoma. These data imply that the expression of  $\alpha v\beta 3$  correlates with the malignant or metastatic form of human melanoma. Based on these results, we investigated the in vitro and in vivo growth properties of M21 human melanoma cells and the adhesion variant M21-L cells that fail to express integrin  $\alpha v\beta 3$  due to the absence of  $\alpha v$  mRNA (10). In this report we demonstrate that M21-L cells show reduced proliferation in vivo, yet in vitro they grow at the same rate as the wild type M21 cells. Transfection of the M21-L cells with the  $\alpha v$  gene not only restores the adhesive capacity of these cells, but also fully restores their proliferative capacity in vivo. These results demonstrate a direct role for an  $\alpha$ v integrin in the growth of human melanoma cells in vivo.

#### Methods

*Cell propagation.* M21 human melanoma cells and their adhesive variants were propagated in RPMI 1640 containing 10% fetal bovine serum as previously described (10). Cells were free of mycoplasma during the course of these studies. For cell growth in vivo, M21 cells or adhesion variants in 0.1 ml PBS, pH 7.2, were injected subcutaneously into the flank of 6-wk-old female nude mice as described (11).

Monoclonal antibodies. MAbs used in these studies included mAbs LM609 (anti- $\alpha\nu\beta3$ ) (10), P3G2 (anti- $\alpha\nu\beta5$ ) (12), and P4C10 (13) or LM534 (anti- $\beta1$  integrins). mAb P3G2 was provided by Drs. D. Horn (Oncogen Science, Inc., Seattle WA) and E. A. Wayner (University of Minnesota), who also provided mAb P4C10. mAb W6/32 directed to HLA class I antigens was obtained from the American Type Culture Collection, Rockville, MD. mAbs specific for  $\alpha2\beta1$ ,  $\alpha3\beta1$ ,  $\alpha4\beta1$ , and  $\alpha5\beta1$  were generously provided by Dr. E. A. Wayner, and anti- $\alpha6\beta1$  was provided by A. Sonnenberg (The Netherlands Red Cross).

Transfection of M21-L cells with  $\alpha v$ . M21-L cells were transfected with 10  $\mu$ g of the plasmid pcDNA I/NEO (Invitrogen) containing  $\alpha v$ cDNA of 4.2 kB (14), using the Lipofectin method (Bethesda Research Laboratories, Bethesda, MD). Transfected cells were cultured in growth medium containing 500  $\mu$ g/ml Geneticin (Sigma Chemical Co., St. Louis, MO) for 6–8 wk. These growth conditions kill all untransfected cells in 1–2 wk. From a panel of transfected subpopulations, cells expressing wild type levels of  $\alpha v$  integrins (M21-L4) and nonexpressing

Address correspondence to Dr. David A. Cheresh, Department of Immunology, Scripps Research Institute, 10666 N. Torrey Pines Road, La Jolla, CA 92037. The present address of Dr. Brunhilde Felding-Habermann is E. Merck Basic Research, Frankfurter Strasse 250, 6100 Darmstadt, Federal Republic of Germany. The present address of Dr. Cynthia A. Romerdahl is BASF Bioresearch, 195 Albany St., Cambridge, Massachusetts 02139.

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control cells (M21-L12) were selected by repeated fluorescence-activated cell sorting using anti- $\alpha$ v mAb LM 142 as described (10). In this case, ~ 10<sup>4</sup>  $\alpha$ v-positive or  $\alpha$ v-negative cells were selected out of 10<sup>6</sup> drug-resistant cells. Northern blot analysis of the transfected cells (M21-L4) revealed an mRNA of ~ 4.2 kb, which is the size of the cDNA used for transfection. In contrast, wild type M21 cells expressed a single mRNA of greater than 7 kb, consistent with previous published results from our laboratory (15).

Cell adhesion assay. Cell adhesion experiments were performed as previously described (12). In brief, <sup>51</sup>Cr-labeled M21 cells were suspended in Hanks' balanced salt solution containing 1% BSA and 1 mM calcium, magnesium, and 0.5 mM manganese, and allowed to attach to 48-well nontissue culture plastic wells (Costar, Data Packaging, Cambridge, MA) coated with individual adhesive ligands (10  $\mu$ g/ml). After adhesion, unbound cells were washed away and adherent cells were harvested and counted as described (12). Cells failed to adhere to BSAcoated wells.

*Immunoprecipitation.* Cell surface integrin expression was monitored by immunoprecipitation of detergent extracts from <sup>125</sup>I-surface labeled cells as previously described (12). In brief, M21 cells were surface labeled, lysed, and subjected to immunoprecipitation using antiintegrin mAbs coupled to affi-gel beads. Immunoprecipitates were analyzed by SDS-PAGE analysis on 7.5% polyacrylamide gels under reducing conditions.

#### Results

To examine the role of  $\alpha$ v-integrins in human melanoma growth in vivo M21 cells or M21-L cells, lacking  $\alpha v$  gene expression, (10) were injected subcutaneously into athymic nude mice. As shown in Fig. 1 A, M21 cells exhibited marked tumorigenicity when compared with M21-L cells. Injection of 10<sup>5</sup> M21 cells produced rapidly growing tumors in 3/5 animals, while animals injected with this number of M21-L cells failed to develop tumors (Fig. 1 A) even after several months. However, after inoculation of  $5 \times 10^5$  M21-L cells, 2/5 animals developed relatively small tumors (Fig. 1 A). This same number of M21 cells lead to rapidly growing and comparatively large tumors in 5/5 animals. It is important to point out that the cells cultured from M21-L tumors did not express  $\alpha v$  integrins, indicating that growth of these tumors did not depend on the in vivo selection of  $\alpha$ v-bearing cells (data not shown). The differences in tumorigenicity of these cells could not be explained by an inherent growth deficiency of the M21-L cell population, since both cell types exhibited identical proliferation rates in vitro (Fig. 1 B). Thus, M21-L cells, lacking functional  $\alpha v$  integrin(s), showed decreased tumorigenicity, suggesting  $\alpha v$  integrin(s) may be fundamentally involved in the malignant phenotype of human melanoma in vivo.

To confirm a direct role for  $\alpha v$  in the tumorigenicity of these melanoma cells, M21-L cells were stably transfected using a vector containing a full-length  $\alpha v$  cDNA and a gene encoding neomycin resistance. After transfection, drug-resistant subpopulations were selected for  $\alpha v$  expression by fluorescence-activated cell sorting. As shown in Fig. 2, M21-L variant-4 (M21-L4) expressed  $\alpha v\beta 3$  and  $\alpha v\beta 5$  at levels similar to the parental M21 cells, while M21-L variant 12 (M21-L12) cells did not express either receptor. These cell lines were not cloned, but rather, sorted for  $\alpha v$  expression based on the selection of 1% of the total cell population. Immunoprecipitation analysis of the expression of  $\alpha v$  confirmed these results (Fig. 3) and revealed that expression of  $\alpha v \beta 3$  than  $\alpha v\beta 5$ , as observed with M21 cells (12). Moreover, M21-L4 cells expressed an mRNA



Figure 1. In vivo and in vitro growth properties of M21 and M21-L cells. (A) M21 cells (closed circles) or M21-L cells (open circles) were injected subcutaneously into athymic nude mice ( $10^5$  cells/animal, *left*, or  $5 \times 10^5$  cells/animal, *right*). The diameter of each tumor was measured with calipers at various times postinjection. Each point represents the mean tumor diameter±SE of all tumor-bearing animals. (B) In vitro growth of M21 (closed circles) and M21-L cells (open circles). Cells were seeded into 24-well plates in RPMI medium containing 10% FCS (Gibco Laboratories, Inc., St. Lawrence, MA). Cells were enumerated with a hemocytometer at various times. Each point represents the mean of triplicate well counts.

of ~ 4.2 kb, which is the size of the cDNA used for transfection (data not shown). In contrast, M21 cells expressed an mRNA of ~ 7 kb (data not shown), consistent with previous published observations (15). M21-L12 cells, like the M21-L cells, showed no detectable  $\alpha v$  integrins, yet all cells expressed equivalent levels of  $\beta 1$  integrins (Figs. 2 and 3). In addition, we examined each of these cell populations using monoclonal antibodies specific for each of the following  $\beta 1$  integrins:  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ , and  $\alpha 6$ . Analysis of these integrins by flow cytometry showed no significant difference in these  $\beta 1$  integrins on any of the four M21 cell lines tested (data not shown).

The adhesive properties of these melanoma cell variants were tested on immobilized ligands. As shown in Fig. 4 (*left*), all cell lines examined attached to collagen I and fibronectin, consistent with their identical expression of  $\beta 1$  integrins. However, cell adhesion to vitronectin was only evident in M21 and the  $\alpha$ v-transfected M21-L4 cell lines. To investigate the mechanism of M21-L4 adhesion to vitronectin, these cells were al-



Figure 2. FACs Analysis of  $\alpha v (\alpha v\beta 3, \alpha v\beta 5)$  and  $\beta 1$  integrins of M21 cell variants. M21-L cells (10) were transfected with 10  $\mu$ g of the plasmid pcDNA I/NEO (Invitrogen) containing  $\alpha v$  cDNA (14), using Lipofectin (Bethesda Research Laboratories, Bethesda, MD). Transfected cells were cultured in 500  $\mu$ g/ml Geneticin (Sigma Chemical Co.) for 8 wk. From a panel of transfected subpopulations, cells expressing wild type levels of  $\alpha v$  integrins (M21-L4) and nonexpressing control cells (M21-L12) were selected by repeated fluorescence-activated cell sorting using anti- $\alpha v$  mAb LM 142 as described (10). In this case,  $\sim 10^4 \alpha v$ -positive or  $\alpha v$ -negative cells were selected out of 10<sup>6</sup> drug-resistant cells. (A) Flow cytometric analyses of M21 cell variants. Primary mAbs used were LM609 (anti- $\alpha v\beta 3$ ) (10), P3G2 (anti- $\alpha v\beta 5$ ) (12), and P4C10 (anti- $\beta 1$  integrins) (22).  $\beta 3$  and  $\beta 5$  integrin profiles in M21-L and M21-L12 cells were identical with non-binding isotype control antibodies.

lowed to attach to this protein in the presence of anti-integrin antibodies. As shown in Fig. 4 (*right*), anti- $\alpha\nu\beta3$  significantly inhibited M21-L4 cell adhesion to vitronectin; however, inclusion of anti- $\alpha\nu\beta5$  was required for complete inhibition consistent with the low level of  $\alpha\nu\beta5$  on these cells. Thus,  $\alpha\nu$  gene expression restored both of these integrins to the surface of M21-L4 cells in a functional form. M21-L4 cells also regained adhesion to fibrinogen and von Willebrand factor (not shown), mediated exclusively by  $\alpha\nu\beta3$  on M21 cells (10).

To test the hypothesis that the expression of the  $\alpha v$  gene product on human melanoma cells plays a critical role in their tumorigenicity, athymic nude mice were injected with M21, M21-L, M21-L4, or M21-L12 cells. As shown in Fig. 5, 10<sup>6</sup> M21 cells, injected subcutaneously, produced rapidly growing tumors in 7/8 mice. In contrast, M21-L cells produced tumors in only 2/8 animals, and these were considerably delayed and smaller in appearance. However, expression of transfected  $\alpha v$ in the M21-L4 cells restored the tumorigenicity: 5/8 animals injected with M21-L4 cells developed tumors, while the nonexpressing variant M21-L12 produced a relatively small tumor in 1/8 animals. These findings were confirmed in three independent experiments, the results of which are compiled in Table I and one experiment in which an increased cell number (2  $\times$  10<sup>6</sup>) was injected (Table I). In the experiments in which 10<sup>6</sup> cells were injected, M21-L and the  $\alpha$ v-negative transfectant M21-L12 cells showed a statistically significant reduction in median tumor volume when compared with the parental M21 cell population (P < 0.01). In contrast, the  $\alpha$ v-positive transfec-



Figure 3. Immunoprecipitation of  $\alpha v$  and  $\beta 1$  integrins from surfacelabeled M21 cell variants. M21, M21L, M21L4, or M21L12 cells (lanes *1-4*, respectively) were surface labeled, lysed with detergent, and subjected to immunoprecipitation with mAb LM609 (*left*, anti- $\alpha v\beta 3$ ); mAb P3G2 (*center*, anti- $\alpha v\beta 5$ ) of mAb LM534 (*right*, anti- $\beta 1$ ) as described in Methods. Immunoprecipitated material was analyzed by SDS-PAGE on a 7.5% gel under nonreducing conditions and autoradiography as described (12). Molecular weight markers are identified on the left.

tants, M21-L4 cells, revealed no significant difference in tumor volume when compared with the M21 cells. The animals injected with  $\alpha v$ -expressing M21 cells and M21-L4 cells showed 12/19 (63%) and 13/21 (62%) tumors, respectively, while the animals injected with  $\alpha v$ -negative M21-L and M21-L12 cells showed tumors in 3/21 (14%) and 2/15 (13%) of the animals, respectively (Table I). These data provide direct evidence for the role of an  $\alpha v$  integrin in the malignant phenotype of human melanoma. That  $\alpha v$  transfection recovered both  $\alpha v\beta 3$  and  $\alpha v\beta 5$  on the cell surface, implies that one or both of these receptors is involved in the tumorigenicity of human melanoma. Although recent findings indicate that  $\alpha v$  can associate



Figure 4. Adhesive properties of M21 cell variants. Attachment to immobilized ligands. (Left) (A) M21 cells ( $\alpha v+$ ), (B) M21-L cells ( $\alpha v-$ ) and the transfectants, (C) M21-L4 cells ( $\alpha v+$ ), and (D) M21-L12 cells ( $\alpha v-$ ) were labeled with <sup>51</sup>Cr, resuspended in adhesion buffer, and 10<sup>5</sup> cells were allowed to attach for 60 min at 37°C to individual wells of a nontissue culture-treated 48-well plate coated with 10 µg/ml vitronectin, fibronectin, and collagen. After, adhesion unbound cells were washed away and the attached cells were harvested as described (12). Cells failed to adhere to bovine serum albumin. Each bar represents the mean±SD of quadruplicates. (Right) M21-L4 cells were allowed to attach to vitronectin-coated wells (as above) in the presence of 50 µg/ml of the control mAb W6/32 (anti-HLA class I antigens (a); mAb LM609 (anti- $\alpha \nu\beta$ 3, (b); mAb P3G2 (anti- $\alpha \nu\beta$ 5, (c); or both antibodies (d). Each bar represents the mean±SD of triplicates.



Figure 5. In vivo growth properties of M21 cell adhesion variants. M21, M21-L, M21-L4, or M21-L12 cells ( $10^6$ ) were injected subcutaneously into the flank of eight female athymic nude mice. At various times, postinjection tumor volumes were measured with calipers and calculated as  $(a^2 \times b)/2$ , with (a) being the width, and (b) the length of the tumor. All data points on each line represent sizes of individual tumors. In vitro growth of all M21 cell variants was indistinguishable (equivalent to Fig. 1 B).

with  $\beta 1$  in some cells (16, 17), we could not detect  $\alpha v\beta 1$  on these cells (data not shown).

#### Discussion

Malignant melanoma is initiated within the skin and is characterized by a proliferative and invasive growth at the primary site. This can ultimately lead to widespread metastatic disease that is characterized by a multistep process that requires not only cell proliferation, but also depends on the ability of the tumor cells to secrete proteases, migrate, promote angiogenesis, and resist host immune functions (18–21). It has been widely hypothesized that the malignant phenotype of melanoma and other human tumors is facilitated by tumor cell matrix interactions. Support for this concept has come from a number of studies indicating that tumor cell surface integrins may be involved in the malignant invasive phenotype of tumor cells (2–8).

Integrin  $\alpha v\beta 3$  is commonly found on melanoma cell lines (10). In fact, this integrin is preferentially expressed on melanoma tissue, including vertically invasive primary as well as

metastatic lesions, while it is absent on normal melanocytes, nevi, and surgically treatable horizontal primary melanoma lesions (9). This tissue distribution indicates that integrin  $\alpha v\beta 3$ is not only a marker of the proliferative and/or invasive phenotype of human malignant phenotype, but that it may also play a role in the biology of human melanoma. To address this issue, we selected an  $\alpha v$ -negative human melanoma cell line using a monoclonal antibody directed to the  $\alpha v$  subunit (10). This cell line, termed M21-L, specifically lacks mRNA-encoding  $\alpha v$ , and thus fails to express integrin  $\alpha v\beta 3$  on its surface (10).

In this report we demonstrate that M21-L cells show dramatically reduced tumorigenicity when injected subcutaneously into athymic nude mice. This is of interest since M21-L cells in vitro grow at the same rate as the M21 parental population. These results indicate that specific growth conditions within the in vivo microenvironment favor cells that express  $\alpha$ v integrin(s). To confirm a direct role of an  $\alpha$ v integrin in the in vivo growth of this cell line, we transfected M21-L cells with a cDNA-encoding  $\alpha v$  and selected cells that express wild-type levels of  $\alpha v\beta 3$  and  $\alpha v\beta 5$  (M21-L4 cells) and a control population selected for the absence of these integrins (M21-L12 cells). M21-L4 cells not only regained their adhesive properties to vitronectin but also showed a completely restored in vivo growth rate, demonstrating a direct role of  $\alpha v$  in this process. It is important to emphasize that the M21 or variant cell lines expressed equivalent levels of  $\beta 1$  integrins and therefore attach effectively to fibronectin, collagen, and laminin. These results indicate that an adhesive ligand in vivo preferentially associates with an  $\alpha v$  integrin, and this may provide an important substrate for attachment and proliferation of human melanoma cells within the microenvironment. Thus, it is possible that vitronectin, a ligand common to both  $\alpha v\beta 3$  and  $\alpha v\beta 5$  (12, 15, 22), may be such a ligand. Alternatively, fibrinogen, von Willebrand factor, or some other ligand known to interact with the promiscuous integrin  $\alpha v\beta 3$  (10) may potentiate melanoma cell proliferation.

In any event, it is apparent that the expression of either  $\alpha v\beta 3$  and/or  $\alpha v\beta 5$  is directly involved in the proliferative response of human melanoma. While M21 cells rapidly proliferate at the primary site these cells do not readily metastasize in athymic nude mouse model, even though they were derived from a metastatic lesion from a patient (23). Metastasis is a multistep cascade that involves proliferation, invasion, and migration. However, with any tumor, metastatic spread is preceded by cellular proliferation at the primary site. Our results

Table I. aV Expression Restores Tumorigenicity of M21-L Cells in Nude Mice

Cell line (aV expression)	M21 (+)	M21-L (-)	M21-L4 (+)	M21-L12 (-)
No. cells s.c. $(10^6)$				
Ratio of mice with tumor take	12/19	3/21	13/21	2/15
Median tumor volume and range (mm <sup>3</sup> )	55.7 (0-675)	$0^*(0-200)$	87 5 <sup>‡</sup> (0-526 5)	0* (0_196)
No. cells s.c. $(2 \times 10^6)$		• (• =••)	07.5 (0-520.5)	0 (0-190)
Ratio of mice with tumor take	7/7	5/7	7/7	4/7
Median tumor volume and range (mm <sup>3</sup> )	600 (220–1328)	294† (0–526)	425 <sup>‡</sup> (269–677)	40* (0–550)

Tumorigenicity of M21 cell variants in the skin of nude mice. Tumors were measured as in Fig. 5. Tumor sizes were compared 21 d after injection. Differences between experimental groups was determined using the Wilcoxon rank sum test. \* Significantly different from M21 values (P < 0.01). † Significantly different from M21 values (P < 0.05). ‡ Not significantly different from M21 values.

demonstrate that it is this initial phase that depends on  $\alpha v$ integrin(s). A recent study demonstrated that expression of  $\alpha 2\beta 1$ , a collagen/laminin receptor on rhabdomyosarcoma, increased the metastatic capacity of those cells without affecting the growth rate of the primary tumor (24). Viewed together, these results imply that specific integrin-ligand interactions may be involved in separate steps of tumor progression leading to metastatic disease.

Our findings are consistent with recent studies, indicating a role for  $\alpha v\beta 3$  in melanoma cell proliferation in vivo. Specifically, an antibody directed to the  $\beta 3$  subunit was shown to reduce the tumorigenicity of human melanoma cells in athymic nude mice (5). In addition, an RGD-containing peptide that can inhibit the function of  $\alpha v$  integrins (15) blocked experimental murine melanoma metastases in vivo (8). The detection of integrin  $\alpha v\beta 3$  on human proliferative melanoma biopsy material (9) and its absence on normal melanoma suggest that the expression of this receptor correlates with the progression of melanoma in situ. Our results support this contention and provide direct evidence that the  $\alpha v$  gene product potentiates the malignant phenotype of human melanoma.

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