[CANCER RESEARCH 50, 6757-6764, October 15, 1990]

Integrin Distribution in Malignant Melanoma: Association of the β_3 Subunit with Tumor Progression[1]

Steven M. Albelda,[2] Stephen A. Mette, David E. Elder, RoseMary Stewart, Laszlo Damjanovich, Meenhard Herlyn, and Clayton A. Buck

Pulmonary Section, Department of Medicine, University of Pennsylvania [S. M. A., S. A. M.]; The Wistar Institute of Anatomy and Biology [S. M. A., S. A. M., L. D., M. H., C. A. B.]; Pigmented Lesion Study Group, Hospital of the University of Pennsylvania [D. E. E., R. S., M. H.]; and Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine [D. E. E.], Philadelphia, Pennsylvania 19104

ABSTRACT

Since tumor progression is dependent on the ability of malignant cells to interact with the extracellular matrix, molecules on the cell surface which mediate cell-substratum interactions are likely to be important regulators of tumor invasion and metastasis. The purpose of this study was to examine the distribution of one such group of cell adhesion receptors, the integrins, in benign and malignant lesions of human melanocytes. The distribution of integrin adhesion receptors was defined on cells in culture derived from normal and malignant melanocytes and in tissue sections from benign to increasingly malignant melanocytic lesions using a panel of monoclonal antibodies against specific integrin subunits. Cells in culture expressed a large variety of integrins, including all of the previously characterized members of the β_1 subfamily plus the α_v/β_3 vitronectin receptor. The expression of integrins was similar in cells cultured from either benign or malignant lesions. In contrast, consistent differences were noted in integrin expression by cells within tissues containing metastatic and vertical growth phase melanomas when compared to radial growth phase melanoma cells and cells within nevi. Most notably, the expression of the β_3 subunit was restricted exclusively to cells within vertical growth phase and metastatic melanomas. The presence of this integrin may be important in the development of tumor invasiveness and could be useful as a marker of melanoma cells entering the more aggressive phase of the malignant process.

INTRODUCTION

The process of tumor invasion and metastasis requires complex changes in normal cell-cell and cell-substratum interactions (1, 2). In order for tumor cells to migrate through adjacent tissues and become invasive, normal cell contacts must be broken and the cells must be able to attach efficiently to the extracellular matrix proteins of the surrounding stroma (1). The cellular receptors mediating these adhesive events are thus likely to be important in tumor invasion and metastasis (2, 3).

One family of cell surface proteins that participates in cell adhesion and migration is the integrins (4-7). Structurally, each integrin is a heterodimer consisting of an α subunit noncovalently associated with a β subunit. The receptor complex spans the plasma membrane, linking the internal cytoskeletal network of a cell with the external extracellular matrix (4). Specificity for ligand binding is determined by the particular combination of α and β subunits. The integrins are divided into at least 5 subfamilies, each being defined by a common β subunit (5). The best characterized subfamilies are the β_1 subfamily, which includes receptors for laminin, fibronectin, and collagen; the β_2 subfamily, found on leukocytes, which includes receptors mediating cell-cell interactions; and the β_3 subfamily, which includes the platelet glycoprotein IIb/IIIa complex and the "vitronectin" receptor which also binds fibrinogen, thrombospondin, and von Willebrand's factor (8, 9).

Data from *in vitro* and *in vivo* comparisons of control and malignant cells have suggested that changes in integrin expression accompany malignant transformation. However, no uniform pattern of change has emerged. Rodent cells transformed with Rous sarcoma virus (10), as well as basal cell or squamous cell carcinomas (11), show a reduction in the expression of integrins from the β_1 subfamily. In contrast, chemically transformed, tumorigenic human osteosarcoma cells display an increase in β_1 integrins when compared to nontumorigenic osteosarcoma cells (12). Other transformed cells show an alteration in the distribution of integrins with no sign of changes in their expression (13).

Despite the lack of consistent changes in the pattern of integrin expression that correlate with tumorigenesis, functional studies suggest that integrins are important in the metastatic process. Synthetic peptides containing the amino acid sequence RGD[3] (derived from one of the cell-binding domains of fibronectin and other matrix proteins) that block the binding of many integrins to their extracellular matrix ligands (14, 15) inhibit the movement of human melanoma cells through an amniotic basement membrane in an experimental model of invasion (16). These peptides also reduce the number of metastatic nodules found within the lungs of mice given B16F10 melanoma cells (17-19). Consistent with these studies is the observation that antibodies against the β_3 subfamily of integrins prevent the establishment of tumors when human melanoma cells are implanted into nude mice (20). Finally, the overexpression of the fibronectin receptor, $\alpha_5\beta_1$, reduces the ability of Chinese hamster ovary cells to form tumors in nude mice (21).

The purpose of this study was to examine the distribution of integrins in a well-characterized system of human tumor progression which results in the development of malignant melanoma (22, 23). A panel of antibodies directed against specific integrin subunits was used to characterize the integrin repertoire of cells from human melanocytic neoplasms in cell culture and in tissue sections. Since cultured cells explanted from all stages of tumor development have been established and characterized (24-27), this approach provided the opportunity to compare integrin expression of malignant cells in culture with those *in situ*, as well as to monitor integrin expression during tumor progression.

MATERIALS AND METHODS

Antibodies. Both Mabs and polyclonal antibodies directed against various subunits of different integrin receptors were used for immuno-

Received 4/17/90; accepted 7/12/90.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants HL-01587 (S. A.) and HL 39023 (C. B.) from the National Heart, Lung and Blood Institute; grants CA-19144 (C. B.), CA10815, CA25294 (D. E.), and CA25874 (M. H.) from the National Cancer Institute; and grants from the W. W. Smith Charitable Trust and American Cancer Society (S. A.).

[2] To whom requests for reprints should be addressed, at 975 Maloney Building/H.U.P., 3600 Spruce Street, Philadelphia, PA 19104.

[3] The abbreviations used are: RGD, Arg-Gly-Asp; Mab, monoclonal antibody; SDS, sodium dodecyl sulfate; RGP, radial growth phase; VGP, vertical growth phase; AEC, aminoethylcarbazole.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/50/20/6757/2441486/cr0500206757.pdf by guest on 24 August 2022

precipitation. Only Mabs were used for tissue staining. Mabs P1H5, P1B5 and P1D6 (recognizing α_2, α_3, and α_5 subunits, respectively) were generously provided by Drs. Elizabeth Wayner and William Carter (28, 29). Dr. Martin Hemler kindly supplied the Mabs TS2/7 and B-5H10 directed against the α_1 and α_4 subunits, respectively (30, 31). Dr. Arnoud Sonnenberg donated the GoH3 monoclonal antibody directed against the α_6 integrin (32). Drs. Joel Bennett and James Hoxie provided the SSA6 Mab that is directed against the β_3 subunit and B1B5 directed against platelet glycoprotein IIb (α_{IIb})(33). The Mab LM142, directed against the vitronectin receptor α subunit (α_v), was donated by Dr. David Cheresh (34). The polyclonal antibody raised against the 140-kDa adhesion receptor complex in rat L6A cells which reacts against integrins in the β1 and β_3 subfamilies has been previously described (35).

Cell Lines. Sixteen different cell lines derived from primary and metastatic melanomas were examined. In addition, four lines of melanocytes explanted from normal skin and congenital nevi were studied. Cultured melanocytes were isolated from foreskins of newborn humans as previously described (26). The human nevus and tumor cells used in these studies have been characterized elsewhere (24–27). Cells were routinely cultured in MCDB153/L-15 medium supplemented with fetal calf serum, pituitary extract, insulin, and transferrin (26). In five cases, melanoma cell lines derived from both the primary melanoma and a metastatic lesion from the same patient were available.

Labeling of Cells. For ^{125}I labeling, intact monolayers of cells in 25-cm^2 tissue culture flasks were washed with phosphate-buffered saline and exposed sequentially to 100 units/ml of lactoperoxidase (Sigma Chemical Co., St. Louis, MO), 1 mCi of carrier-free ^{125}I (Amersham, Arlington Heights, IL), and three 40-μl aliquots of 0.06% hydrogen peroxide. The cells were harvested and extracted as described below.

Cell Harvest and Nonidet P-40 Extractions. After being washed three times with phosphate-buffered saline, labeled cells were extracted by exposing the monolayers to 0.5–1.0 ml of 0.01 M Tris acetate buffer, pH 8.0, containing 0.5% Nonidet P-40, 0.5 mM Ca^{2+}, and phenylmethylsulfonyl fluoride at a 2 mM concentration. Cells were then scraped from the vessel and the extraction continued for 20 min at 4°C. The extract was centrifuged for 30 min at 12,000 × *g* and the resulting supernatant used for immunoprecipitation.

Immunoprecipitation and Gel Electrophoresis. Nonionic detergent extracts were preadsorbed for 30 min at 4°C with protein G conjugated to Sepharose beads (Pharmacia, Piscataway, NJ). Fifty μl of the appropriate antibody was then added to 100 μl of the extract and the mixture allowed to stand for 1 h at 4°C. Immunocomplexes were collected by adsorption onto protein G Sepharose beads for 1 h at 4°C. The beads containing the complex were washed 5 times with a buffer containing 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5% deoxycholate, and 0.1% SDS. The beads were then suspended in an electrophoresis sample buffer [62.5 mM Tris base, 2% SDS, 10% glycerol (pH 6.8)]. The material eluted from the beads was analyzed by SDS-polyacrylamide gel electrophoresis using 6% polyacrylamide gels as previously described (35) under nonreducing conditions. Gels were dried and exposed to Kodak XR-5 X-ray film at −70°C.

Origin and Classification of Tissue Samples. All tissue samples were obtained from diagnostic or therapeutic biopsies taken from patients in the Pigmented Lesion Group, Hospital of the University of Pennsylvania. The samples were snap frozen in liquid nitrogen and stored at −70°C until the time of sectioning. The biopsy specimens were classified by standard histological criteria (22, 23, 36) as belonging to one of the following groups: benign nevus, primary melanoma (either radial or vertical growth phase), or metastatic melanoma. Nevus cells seen in the benign melanocytic nevus were found as collections of cuboidal cells either in the dermis or at the dermal-epidermal junction without any suggestion of nuclear atypia or inflammatory response. Primary melanomas were divided into RGP or VGP (22, 23, 36). RGP melanomas were defined as proliferations in the epidermis of moderately to severely atypical melanocytic cells with or without small clusters of lesional cells in the dermis without proliferation. The VGP primary melanomas were invasive into the dermis in proliferative clusters or in sheets and tended to show greater atypia than RGP cells. The VGP melanomas formed clinically and histologically recognizable tumor masses at the primary site and may therefore be considered "tumorigenic" primary melanomas. Metastatic melanomas resembled the VGP cells both histologically and cytologically, but these tumorigenic lesions were found at sites distant from the primary lesion. Some specimens revealed both the RGP and VGP compartments. In these cases, each portion of the lesion was analyzed separately.

Immunohistochemistry. Cryostat sections 4- to 8-μm thick were placed on poly-L-lysine-coated slides and fixed in −20°C acetone for 10 min. The peroxidase-based Vectastain Elite ABC system (Vector Laboratories, Burlingame, CA) was used to detect the monoclonal anti-integrin antibodies. In lesions with large amounts of brown melanin pigment, the red AEC substrate was used for contrast. These sections were lightly counterstained with Mayer's hematoxylin. For the less pigmented nevus cells, diaminobenzidine was used as the the peroxidase enzyme substrate without the use of counterstaining. Each lesion was classified using the criteria described above and the degree of immunoperoxidase staining of the melanocytes or melanoma cells was graded negative, weak staining, or strong staining. Staining was considered positive for an entire lesion if 10% or more of the cells showed strong staining. For reference purposes, the staining shown by the nevus cells in Fig. 2, *F* and *G*, were graded as strongly positive, the nevus cells in Fig. 2, *C* and *D*, as weakly positive, and the nevus cells in Fig. 2, *H* and *I*, as negative. The percentage of cells positive with any of the monoclonal antibodies was visually estimated. Color slides were taken using Ektachrome 64 indoor film. For black and white pictures, Kodak T-MAX 400 film was used.

Statistical Analysis. The proportion of tumorigenic *versus* nontumorigenic lesions expressing β_3 and α_4 integrin subunits were analyzed using χ^2 analysis.

RESULTS

Integrin Expression by Cultured Melanocytes and Melanoma Cells. To determine the integrin profile of melanocytes and melanoma cells in culture, extracts of ^{125}I-labeled cell of four lines of normal melanocytes (established from fetal foreskin and benign nevi) and 16 different tumor lines (isolated from primary or metastatic melanomas) were subjected to immunoprecipitation. Examples of immunoprecipitations from one nonmalignant melanocyte line (nevus cell line 1692) and one malignant melanoma cell line (line 164) are shown in Fig. 1. Two radioactive bands are seen in the immunoprecipitations using monoclonal antibodies since antibodies specific for one integrin subunit coprecipitate the associated subunit. Thus, monoclonal antibodies against specific integrin α subunits coprecipitated the common 120-kDa β_1 subunit. Similarly, an anti-α_v subunit monoclonal antibody coimmunoprecipitated the 95-kDa β_3 subunit (data not shown) and the anti-β_3 monoclonal antibody coprecipitated the 150-kDa α_v subunit. In the case of nevus 1692 (Fig. 1, *top*), the major integrins detected were $\alpha_1\beta_1$, a collagen/laminin receptor; $\alpha_3\beta_1$, a promiscuous receptor for laminin, fibronectin, and collagen; $\alpha_5\beta_1$, a fibronectin receptor; and $\alpha_v\beta_3$, a vitronectin receptor. No reactivity with an antibody directed against the platelet glycoprotein IIb was detected. Cultured cells derived from the metastatic melanoma 164 expressed more integrins (Fig. 1, *bottom*) than did the nevus cell line 1692, including all the integrins found on the melanocytes plus the $\alpha_2\beta_1$ collagen/laminin receptor and $\alpha_4\beta_1$, a receptor shown to bind to a non-RGD containing region of fibronectin (37) and function in cell-cell interactions (38).

These comparisons suggested that differences between melanocytes and melanoma cells in culture might exist. However, when the immunoprecipitation data from all 20 cell lines were examined (Table 1), no consistent differences between benign and malignant cells types could be detected. There was clearly

Downloaded from http://aacrjournals.org/cancerres/article-pdf/50/20/6757/2441486/cr0500206757.pdf by guest on 24 August 2022

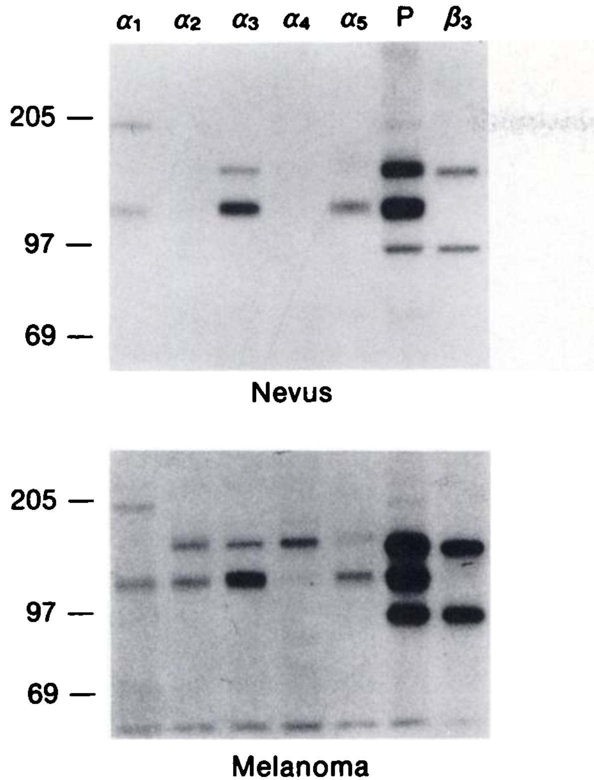

Fig. 1. Immunoprecipitation of integrin subunits from cultured melanocytes and malignant melanoma cells. Nonionic detergent extracts from ^{125}I-labeled melanocytes derived from a nevus (*top*) or a metastatic melanoma (*bottom*) were immunoprecipitated as described in the "Materials and Methods" using monoclonal antibodies directed against five integrin α subunits or the β_3 subunit. Lane *P* (Pan-integrin), immunoprecipitation with a polyclonal antibody which reacts with all integrins from the β_1 and β_3 subfamilies. Each lane is designated according to the integrin α or β subunit that reacted with the monoclonal antibody used in the immunoprecipitation. *Ordinate,* positions and size of molecular mass markers in kDa.

Table 1 *Integrin expression in cell lines*

The distribution of integrin expression in cell lines derived from various types of melanocytic tissues was determined by immunoprecipitation of extracts from ^{125}I-labeled cells using a panel of antibodies. The amount of integrin expression was graded as being present in large amounts (●), being present in trace amounts (*), or being nondetectable (○).

Tissue	Integrin subunit						Anti-VNR[a]
	α_1	α_2	α_3	α_4	α_5	β_3	
Melanocyte							
FM 713	*	○	●	○	*	●	●
FM 624	*	*	●	ND	○	●	●
Nevus							
1692[b]	●	○	●	○	●	●	●
1559	*	*	●	ND	*	●	●
Melanoma							
278 (P)[c]	●	*	●	●	*	●	●
1617 (M)	●	●	●	●	●	●	●
983A (P)[c]	●	*	●	●	*	●	●
983B (M)	●	*	●	*	*	●	●
115 (P)[c]	○	*	●	●	○	●	●
239A (M)	●	*	●	●	*	●	●
1361A (P)[c]	○	*	●	*	*	●	●
1361C (M)	●	●	●	●	*	●	●
75 (P)[c]	●	*	●	*	*	●	●
373 (M)	●	*	●	○	*	●	●
902B (P)	●	○	●	*	○	●	●
793 (P)	*	○	●	●	○	●	●
164[d] (M)	●	●	●	●	●	●	●
451 (M)	○	*	●	*	○	●	●
852 (M)	●	●	●	*	*	●	●
853-2 (M)	●	*	●	●	○	●	●

[a] Anti-VNR, polyclonal antibody against the vitronectin receptor; ND, not determined; P, cells explanted from primary malignant melanomas; M, cells explanted from metastatic malignant melanomas.
[b] Nevus cell line pictured in Fig. 1.
[c] Cell lines explanted from a primary and malignant lesion obtained from the same patient.
[d] Melanoma cell line pictured in Fig. 1.

a high degree of heterogeneity in integrin expression among cell lines. The principal integrins expressed by all the cultured cells were $\alpha_3\beta_1$ and $\alpha_v\beta_3$. The $\alpha_1\beta_1$ collagen/laminin receptor was expressed by many of the melanoma cells and by one of the nevus cell lines at relatively high levels. There was considerable variation in the expression of other integrins. Most cells expressed only trace levels of the classical fibronectin receptor, $\alpha_5\beta_1$, or the collagen/laminin receptor, $\alpha_2\beta_1$. The frequent expression of $\alpha_4\beta_1$ by melanoma cells is particularly interesting, since this integrin has been shown to be involved in cell-cell interactions and could thus possibly play a role in endothelial cell adhesion and extravasation of metastatic cells.

Expression of Integrins on Normal and Malignant Cells in Tissue Sections. The fact that no consistent differences between tumorigenic and nontumorigenic cells in culture were noted did not rule out the possibility that such a difference existed in tissue. Therefore, the integrin profiles of melanocytes and melanoma cells in tissue were compared by immunoperoxidase staining of cryostat sections from 42 different lesions representative of different stages of melanoma progression including benign nevi, RGP primary, VGP primary, and metastatic melanoma (see "Materials and Methods" for details of tumor classification).

The integrins expressed by cells within each cryostat section were determined using antibodies specific for the α subunit of 6 receptors in the β_1 integrin subfamily, the α subunit of the vitronectin receptor (α_v), and the β_3 subunit. An example of the staining pattern of skin containing a benign congenital nevus is illustrated in Fig. 2. The staining in the epidermis (*arrows*) was primarily confined to the basal layers. The keratinocytes stained strongly for the α_2 (Fig. 2*B*), α_3 (Fig. 2*C*), and α_6 (Fig. 2*F*) subunits. There was some expression of α_1 (Fig. 2*A*) and α_v (Fig. 2*G*), but staining with antibodies against α_4 (Fig. 2*D*), α_5 (Fig. 2*E*), and β_3 (Fig. 2*H*) did not exceed the background noted in control sections stained with an antibody against an irrelevant antigen (Fig. 2*I*).

The staining pattern of a benign melanocytic nevus (an example of one of six benign melanocytic nevi studied in detail) is also seen in the same sections (Fig. 2). The nevus cells (*arrowheads*) strongly expressed α_6 (Fig. 2*F*) and α_v (Fig. 2*G*). The expression of α_1 (Fig. 2*A*) was less intense and roughly equivalent to that of the basal keratinocytes in the same section. Some expression of α_2, α_3, and α_4 was noted (Fig. 2, *B, C,* and *D*), but staining was less intensive and patchy in its distribution. On average, 40% of the cells within a given nevus ($n = 6$) were graded as positive for α_2 and 28% for α_3. The nevus illustrated in Fig. 2 was the only one of six to show α_4 staining, with approximately 30% of the nevus cells in the deeper regions of the dermis being positive. Staining of sections from all six nevi for α_5 and β_3 (Fig. 2, *E* and *H*) did not exceed that of the nonspecific background noted in the control sections (Fig. 2*I*).

In general, the expression of the β_1 integrins by RGP ($n = 6$), VGP ($n = 5$), and metastatic cells ($n = 5$) was qualitatively similar to that of nevus cells (data not shown). The α_1 and α_6 subunits were consistently present on all of the malignant cells. The expression of the α_2 and α_3 subunits remained somewhat

Downloaded from http://aacrjournals.org/cancerres/article-pdf/50/20/6757/2441486/cr0500206757.pdf by guest on 24 August 2022

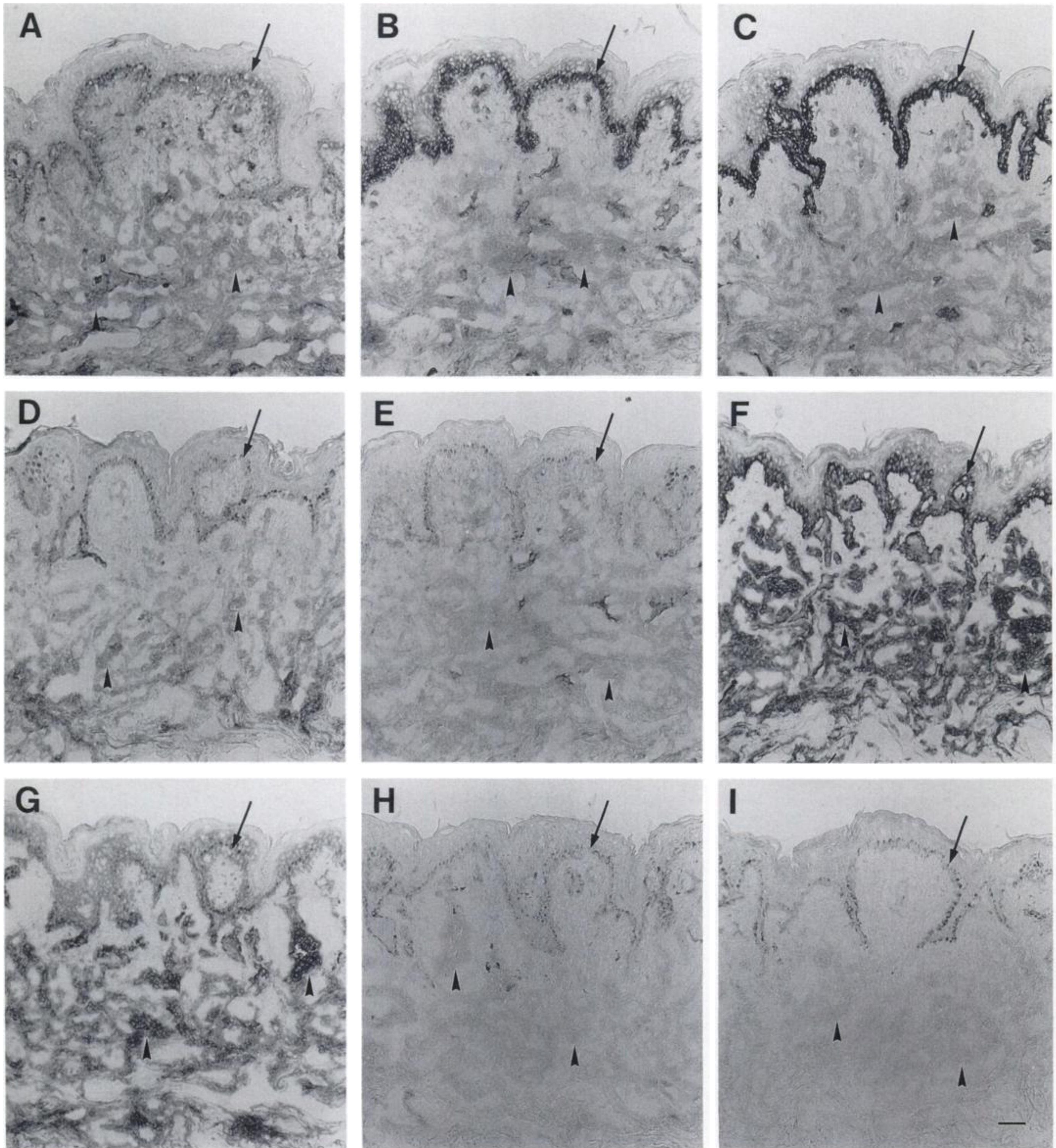

Fig. 2. Tissue distribution of integrins in normal skin and a congenital nevus. Frozen sections of a portion of skin containing a congenital nevus were exposed to monoclonal antibodies directed against one of six α subunits in the β_1 subfamily of integrins, the α_v and β_3 subunits, and a control antibody. The sections were then treated with biotinylated anti-mouse IgG, exposed to avidin-peroxidase complexes, and reacted with diaminobenzadine as the chromagen to identify cells reacting with the monoclonal antibodies. No counterstaining was used. The staining pattern of the basal layer of keratinocytes in the epidermis (*arrows*) can be contrasted with that of the nevus cells which are infiltrating the underlying dermis (*arrowheads*). *Bar*, 100 μm. *A*, anti-α_1; *B*, anti-α_2; *C*, anti-α_3; *D*, anti-α_4; *E*, anti-α_5; *F*, anti-α_6; *G*, anti-α_v; *H*, anti-β_3; *I*, control. Dark areas at the basal layer of the epidermis in sections *D*, *E*, *H*, and *I* (*arrows*) represent melanin deposition in contrast to the diffuse cellular staining seen in sections *A*, *B*, *C*, *F*, and *G*.

heterogeneous, but the percentage of cells within a given lesion that were positive for the α_2 (78% of cells) and α_3 (73% of cells) subunits was higher than in the nevus tissues. The α_5 fibronectin receptor was found in only three samples (two VGP lesions and one metastasis). There appeared to be a difference between the nontumorigenic (nevus and RGP melanomas) and tumorigenic (VGP and metastatic melanomas) lesions with regard to the expression of the α_4 subunit. On our initial survey, the α_4 subunit was expressed in a focal distribution on approximately half of the VGP and metastatic tumors but on only one RGP

Downloaded from http://aacrjournals.org/cancerres/article-pdf/50/20/6757/2441486/cr0500206757.pdf by guest on 24 August 2022

melanoma and one of the nevi. Because of this difference, we screened an additional 6 nevi, 7 VGP lesions, and 6 metastatic tumors for the presence of the α_4 subunit. As shown in Table 2, only 2 of 18 (11%) of the nontumorigenic lesions expressed the α_4 subunit as compared with 9 of 23 (39%) tumorigenic neoplasms. This difference was significant at the $P < 0.05$ level.

The most striking difference noted between tumorigenic and nontumorigenic lesions was the marked contrast in expression of the β_3 subunit. Whereas the cells from nevi, RGP, VGP, and metastatic lesions all stained strongly with anti-α_v antibody, only tumorigenic VGP and metastatic melanoma cells expressed the β_3 subunit. The benign melanocytic nevus and nontumorigenic RGP primary melanoma uniformly failed to express this subunit. Sections selected from each of these pathological classifications, stained with a monoclonal antibody (SSA6) specific for the β_3 subunit are shown in Fig. 3. The loosely packed cluster of cells within the nevus failed to react with this antibody (Fig. 3*A*). Likewise, the cluster of melanoma cells within the nontumorigenic RGP primary melanoma did not react with this antibody (Fig. 3*B*). In marked contrast, VGP cells and metastatic cells stained strongly with this antibody (Fig. 3, *C* and *D*). No staining was observed using an antibody against the α_{IIb} subunit which is found in association with β_3 in platelets (data not shown).

Fig. 4*A* is an overview of a complex primary melanoma that contained regions of invasive VGP cells (*left*), as well as regions of RGP cells (*right*), confined to the epidermis and papillary dermis. The positive reaction with the anti-β_3 monoclonal antibody was evidenced in the areas showing red and was limited to cells within the VGP portion of the tumor. The red of the positive AEC-staining cells could easily be distinguished from the brown of the melanin-containing tumor cells. Although many of these VGP cells expressed the β_3 integrin receptor, heterogeneity of staining was apparent. At lower magnification (Fig. 4*A*) certain regions within the VGP portion of the lesion were clearly reactive with antibodies against β_3 integrins, while other regions showed more heterogeneity in their staining pattern. When examined at higher magnification (Fig. 4*B*), heterogeneity was still apparent, but positively staining cells could be readily detected. This heterogeneity was also seen in other lesions examined. Between 30 and 90% of the cells within a single VGP or metastatic lesion reacted with monoclonal antibodies specific for β_3 integrins. All cells within the RGP portions of this same lesion (Fig. 4*C*) consistently failed to show any reactivity with the anti-β_3 Mab. The brown is due to the melanin produced by these cells.

Because of the striking differences in β_3 expression between nontumorigenic and tumorigenic melanocytes, we expanded our initial survey to include 42 lesions. These results are sum-

Table 2 *Expression of integrin subunits in melanocytic tissue*

Tissue	Integrin subunit	
	α_4	β_3
Nevus	1/6 (30)[a]	0/9
RGP primary melanoma	1/12 (80)	0/12
VGP primary melanoma	4/12 (55 ± 8)	8/10 (61 ± 9)
Metastatic melanoma	5/11 (78 ± 6)	11/11 (66 ± 15)

[a] Numerator, number of lesions where at least 10% of the cells demonstrated strong staining by immunohistocytochemistry; denominator, number of lesions examined; numbers in parentheses, percentage of cells (±SD) within each lesion that reacted with a given antibody.

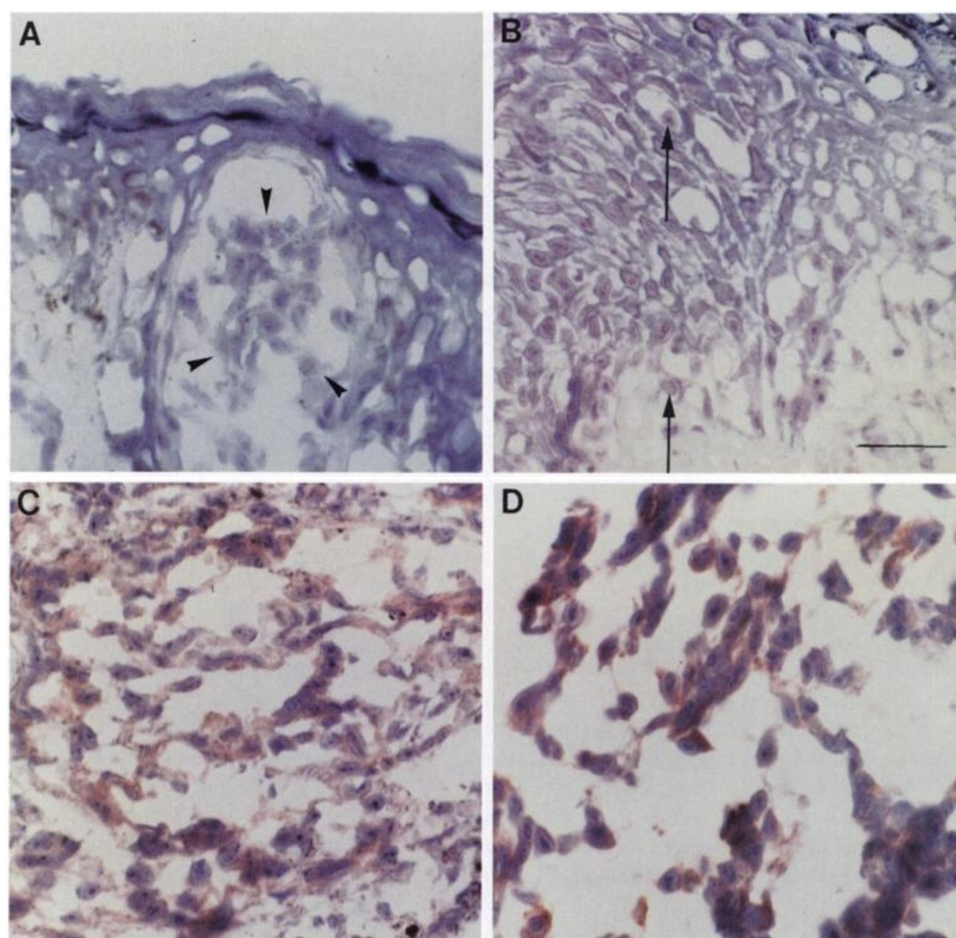

Fig. 3. Tissue distribution of β_3 integrins in melanotic lesions. Frozen sections were stained with an anti-β_3 integrin subunit monoclonal antibody and counterstained by the immunoperoxidase technique using the red AEC chromagen to distinguish reactivity from the brown of melanin (see "Materials and Methods"). *Bar*, 100 µm. *A*, congenital nevus (*arrowheads*); *B*, radial growth phase of a lentigo maligna melanoma containing keratinocytes and and a few neoplastic melanocytes (*arrows*); *C*, vertical growth phase primary melanoma; *D*, metastatic melanoma. There is no reactivity against nevus cells, radial growth phase melanoma cells, or keratinocytes (*A* and *B*). In contrast, vertical growth phase cells and metastatic melanoma cells show evidence of red indicating reactivity with the anti-β_3 monoclonal antibody (*C* and *D*).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/50/20/6757/2441486/cr0500206757.pdf by guest on 24 August 2022

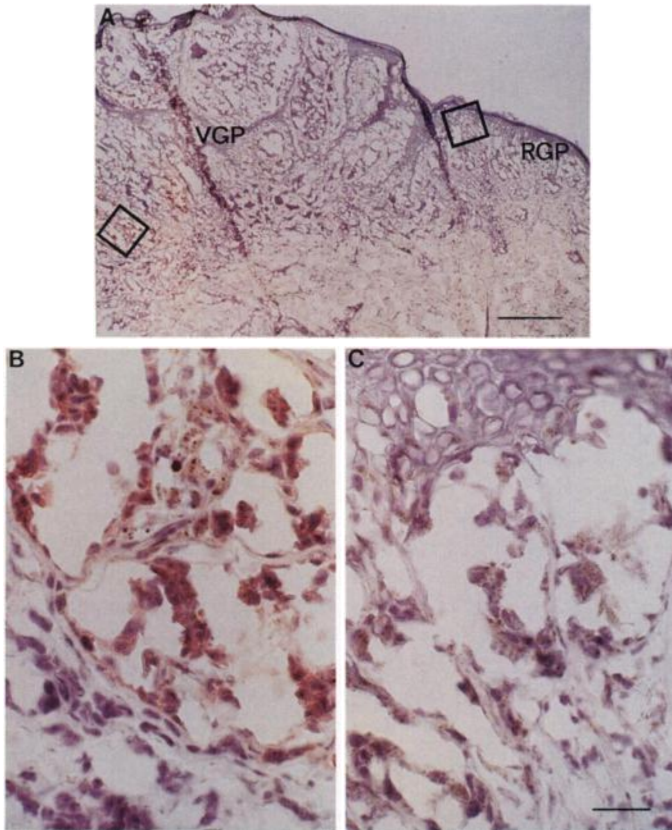

Fig. 4. Distribution of β_3 integrins within a complex primary melanoma. Frozen sections of a complex primary melanoma were stained with the anti-β_3 monoclonal antibody and counterstained by the immunoperoxidase technique using the red AEC chromagen (see "Materials and Methods") to contrast with the brown melanin pigment. In *A*, lesion presents with a VGP nodule that elevates the epidermis (*left*). The nodule is contiguous with a flat RGP (*right*); *bars*, 500 μm. *Insets*, approximate location of fields depicted in Fig. 3, *B* and *C*; *bar*, 100 μm. In *B*, VGP cells contain both brown melanin and red AEC chromagen, indicating reactivity with the anti-β_3 monoclonal antibody. In *C*, RGP cells contain only brown melanin pigment and no red, indicating negative reactivity with the anti-β_3 Mab.

marized in Table 2. Zero of 9 nevi and 0 of 12 RGP lesions expressed the β_3 subunit. In contrast, 8 of 10 VGP lesions (80%) and 11 of 11 (100%) metastatic melanomas stained positively with anti-β_3 antibody. The difference between tumorigenic *versus* nontumorigenic β_3 integrin expression was highly significant ($P < 0.001$).

DISCUSSION

The interaction of tumor cells with extracellular matrix has long been suspected as an important component of tumorigenicity and metastasis (reviewed in Refs. 1 and 3). A comparative analysis of specific cell-matrix adhesion receptors, such as the integrins, present on tumor cells and nontransformed cells may thus be important to our understanding of tumor progression. A number of investigators have begun to study the types and functions of integrins on a variety of tumor cells in culture and have found various changes in integrin expression or function (see "Introduction"). However, to date, no studies have comprehensively examined the distribution of integrins in a well-defined system of tumor progression or compared *in vitro* to *in situ* integrin expression. Because the development of melanomas takes place in well-defined steps (22, 23), and these cells are easily grown in culture, this system is ideally suited for meaningful comparative studies of integrin expression.

Melanocytes and melanoma cells in culture expressed a wide variety of integrins. These included all of the known β_1 or VLA subfamily of integrins plus α_v/β_3. Examination of Table 1 highlights the heterogeneity of integrin expression observed among various cell lines. Although all cells produced readily detectable amounts of the the α_3, α_v, and β_3 subunits, the expression of other integrins was quite variable. Because of this heterogeneity, it was difficult to identify clear differences between the integrin repertoire of melanocytes derived from normal skin and nevi compared with those derived from primary or metastatic melanomas, with the possible exception of an increased expression of the α_4 subunit on cells derived from malignant tumors. Although the significance of this difference is unclear due to the limited number of nonmalignant cell lines studied, the presence of the α_4/β_1 integrin on melanoma cells in culture and in tissues is especially interesting, since this receptor (also known as VLA-4) has been implicated in cell-cell adhesion. The ligand for VLA-4 is an endothelial cell surface protein, VCAM-1 (vascular cell adhesion molecule-1) (37, 38), that appears to be similar or identical to INCAM-110, a recently described inducible endothelial cell surface glycoprotein that mediates the adhesion of certain human melanoma cell lines to cultured endothelium (39). The presence of $\alpha_4\beta_1$ on some of the cultured human melanoma cell lines studied here and on about one-third of the malignant melanoma cells in tissues supports the idea that it may play a role in the metastatic process, although the absence of this receptor on many cell lines and tumors indicates that it is not necessary for metastasis.

Some of the integrins reported here have also been noted by others studying individual melanoma cell lines, both human and murine in origin. Among the integrins expressed in these lines were α_1/β_1 and α_2/β_1 (40), α_4/β_1 (41), and α_6/β_1 (42), as well as the β_3 and α_v subunits (8). These results, taken with those reported here, support the diverse pattern of integrins expressed in culture.

Since the expression of many cell surface proteins is markedly altered by the process of tissue culture (26), it was important to establish the relationship between the distribution of of integrins on cells in culture *versus* those that reside in tissue. The most important finding of this study was the contrast in expression of the β_3 integrins by benign melanocytes and melanomas in the radial growth phase (nontumorigenic lesions) *versus* those melanoma cells in tumorigenic lesions, *i.e.*, the vertical growth phase of primary melanoma or metastatic tumors. Cells within almost all of the VGP and metastatic lesions expressed the β_3 integrin, while benign melanocytes and cells within RGP melanomas did not. In every positive case, between 30 and 90% of cells within a given lesion stained strongly for the β_3 subunit. This pattern of heterogenous expression was in contrast to the more uniform expression of the β_1 subfamily of integrins by epidermal cells but was similar to the the patchy distribution of the α_2 and α_3 subunits by melanomas and melanocytes. Although the reason for the heterogeneity of β_3 receptor expression is not known, heterogenous expression of tumor antigens has been well described in malignant melanoma (43) and many other tumors (44, 45).

These findings agree with and extend those of McGregor *et al.* (46) who found binding of an antibody directed against the platelet glycoprotein IIb/IIIa complex in 16 of 21 (75%) frozen melanoma tissues compared with no binding in 15 cryostat sections containing normal melanocytes in skin or nevi. Since glycoprotein IIb is found exclusively on platelets, it seems likely that the antibody used by these investigators was identifying

Downloaded from http://aacrjournals.org/cancerres/article-pdf/50/20/6757/2441486/cr0500206757.pdf by guest on 24 August 2022

the β_3 subunit. Taken together, these observations emphasize the importance of studying the distribution of cell surface receptors, such as the integrins, on cells in their "natural," *i.e., in situ,* environment, and support the observation made here that the expression of the β_3 subunit is common in invasive melanomas.

RGP and VGP tumors behave in a well-defined and predictable manner (47). The radial growth phase is characterized by indolent but inexorable growth and by a propensity to progress to vertical growth phase. Although RGP melanomas may be invasive, they do not form nodules in the dermis, they do not metastasize to distant sites, and they are not capable of causing metastatic tumors in nude mice (36, 47). Survival, upon excision of tumors at this stage is 100%. Clinically, only those melanomas that express VGP characteristics have the capacity for metastasis (47). Since the distinction between early VGP and RGP can be difficult, the expression of the β_3 subfamily of integrins by melanoma cells with tumorigenic potential could be useful as a marker of cells entering this phase of malignant progression.

In contrast to the selective expression of the β_3 subunit by VGP and metastatic melanomas, the α_v subunit was strongly expressed on all tissues (Fig. 2G). This suggests that the α_v subunit associates with a different β subunit in normal melanocytes and nevi. The ability of the α_v subunit to combine with alternative β subunits is well documented (48–50). One notable difference between the α_v/β_3 receptor and the other α_v-containing receptors is that only the $\alpha_v\beta_3$ receptor has the ability to bind to fibrinogen. This property may somehow be important in the process of tumor progression.

Whether the expression of a receptor containing the β_3 subunit contributes to the capacity of melanoma cells to invade matrix and blood vessels is not known. It is possible, however, that the possession of this additional adhesion receptor allows the cells to move more freely within the mesenchymal stroma during tumor formation and invasion or bind more readily to the endothelial lining of blood vessels during the metastatic process. Evidence that this integrin subfamily may be important in tumor implantation and growth has recently been provided by Boukerche *et al.* (20) who have inhibited the growth of a human melanoma cell line in nude mice using an antibody with reactivity against the β_3 subunit of the vitronectin receptor. Further evidence that the $\alpha_v\beta_3$ receptor may be important in metastasis is provided by studies which have shown that peptides containing the RGD sequence are able to inhibit both melanoma tumor cell invasion (16) and the development of experimental metastases by murine melanomas (17–19).

In summary, this study has demonstrated that melanoma cells in culture and in tissue express a wide variety of integrins in a relatively heterogenous pattern. However, the expression of the β_3 subunit in tissue sections was restricted exclusively to tumorigenic melanoma cells. This suggests that the presence of this receptor may be important for the development of tumor invasiveness and could be useful as a marker of melanoma cells entering the more aggressive phase of the malignant process.

ACKNOWLEDGMENTS

The authors would like to thank Mildred Daise for her excellent technical assistance and Marie Lennon for her skillful preparation of the manuscript. We would also like to express our appreciation to Drs. Elizabeth Wayner, William Carter, Martin Hemler, Arnoud Sonnenberg, Joel Bennett, James Hoxie, and David Cheresh for their generous donations of antibodies.

REFERENCES

1. Liotta, L. A. Tumor invasion and metastases—role of the extracellular matrix: Rhoads memorial award lecture. Cancer Res., *46:* 1–7, 1986.
2. Juliano, R. Membrane receptors for extracellular matrix macromolecules: relationship to cell adhesion and tumor metastasis. Biochim. Biophys. Acta, *907:* 261–278, 1987.
3. Ruoslahti, E., and Giancotti, F. G. Integrins and tumor cell dissemination. Cancer Cells, *1:* 119–126, 1989.
4. Buck, C. A., and Horwitz, A. F. Cell surface receptors for extracellular matrix molecules. Annu. Rev. Cell Biol., *13:* 179- 205, 1987.
5. Hynes, R. O. Integrins: a family of cell surface receptors. Cell, *48:* 549–554, 1987.
6. Ruoslahti, E., and Pierschbacher, M. D. New perspectives in cell adhesion: RGD and integrins. Science (Washington DC), *238:* 491–497, 1987.
7. Albelda, S. M., and Buck, C. A. Integrins and other cell adhesion molecules. FASEB J., *4:* 2868–2880, 1990.
8. Cheresh, D. A., and Spiro, R. C. Biosynthetic and functional properties of an Arg-Gly-Asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen, and von Willebrand factor. J. Biol. Chem., *262:* 17703–17711, 1987.
9. Lawler, J., Weinstein, R, and Hynes, R. O. Cell attachment to thrombospondin: the role of ARG-GLY-ASP, calcium, and integrin receptors. J. Cell Biol., *107:* 2351–2361, 1988.
10. Plantefaber, L. C., and Hynes, R. O. Changes in integrin receptors on oncogenically transformed cells. Cell, *56:* 281–290, 1989.
11. Peltonen, J., Larjava, H., Jaakkola, S., Gralnick, H., Akiyama, S. K., Yamada, S. S., Yamada, K. M., and Uitto, J. Localization of integrin receptors for fibronectin, collagen, and laminin in human skin. J. Clin. Invest., *84:* 1916–1923, 1989.
12. Dedhar, S., and Saulnier, R. Alterations in integrin receptor expression on chemically transformed human cells: specific enhancement of laminin and collagen receptors. J. Cell Biol., *110:* 481–489, 1990.
13. Akiyama, S. K., Larjava, H., and Yamada, K. M. Differences in the biosynthesis and localization of the fibronectin receptor in normal and transformed cultured human cells. Cancer Res., *50:* 1601–1607, 1990.
14. Pierschbacher, M. D., and Ruoslahti, E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature (London), *309:* 30–33, 1984.
15. Yamada, K. M., and Kennedy, D. W. Dualistic nature of adhesive protein function: fibronectin and its biologically active peptide fragments can autoinhibit fibronectin function. J. Cell Biol., *99:* 29–36, 1984.
16. Gehlsen, K. R., Argraves, W. S., Pierschbacher, M. D., and Ruoslahti, E. Inhibition of tumor cell invasion by Arg-Gly-Asp-containing synthetic peptides. J. Cell Biol., *106:* 925- 930, 1988.
17. Humphries, M. J., Olden, K., and Yamada, K. A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. Science (Washington DC), *233:* 467–470, 1986.
18. Saiki, I., Murata, J., Lida, J., Nishi, N., Sugimura, K., and Azuma, I. The inhibition of murine lung metastasis by synthetic polypeptides [poly(arg-gly-asp) and poly(tyr-ile-gly-ser-arg)] with a core sequence of cell adhesion molecules. Br. J. Cancer, *59:* 194–197, 1988.
19. Humphries, M. J., Yamada, K. M., and Olden, K. Investigation of the biological effects of anti-cell adhesion synthetic peptides that inhibit experimental metastasis of B16-F10 murine melanoma cells. J. Clin. Invest., *81:* 782–790, 1988.
20. Boukerche, H., Berthier-Vergnes, O., Bailly, M., Dore, J. F., Leung, L. K., and McGregor, J. L. A monoclonal antibody (LYP18) directed against the blood platelet glycoprotein IIb/IIIa complex inhibits human melanoma growth *in vivo.* Blood, *74:* 909–912, 1989.
21. Giancotti, F. G., and Ruoslahti, E. Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. Cell, *60:* 849–859, 1990.
22. Clark, W., Elder, D., Guerry, D., Epstein, M., Greene, M., and VanHorn, M. A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma. Hum. Pathol., *15:* 1147–1165, 1984.
23. Clark, W., Elder, D., and VanHorn, M. The biologic forms of malignant melanoma. Hum. Pathol., *17:* 443–452, 1986.
24. Herlyn, M., Thurin, J., Balaban, G., Bennicelli, J., Herlyn, D., Elder, D., Bondi, E., Guerry, D., Nowell, P., Clark, W., and Koprowski, H. Characteristics of cultured human melanocytes isolated from different stages of tumor progression. Cancer Res., *45:* 5670–5676, 1985.
25. Balaban, G., Herlyn, M., Clark, W., and Nowell, P. Karyotypic evolution in human malignant melanoma. Cancer Genet. Cytogenet., *19:* 113–122, 1986.
26. Herlyn, M., Rodeck, U., Mancianti, M., Cardillo, F., Lang, A., Ross, A., Jambrosic, J., and Koprowski, H. Expression of melanoma- associated antigens in rapidly dividing human melanocytes in culture. Cancer Res., *47:* 3057–3061, 1987.
27. Rodeck, U., Herlyn, M., Manssen, H., Furlanetto, R., and Koprowski, H. Metastatic but not primary melanoma cell lines grow *in vitro* independently of exogenous growth factors. Int. J. Cancer, *40:* 687–690, 1987.
28. Wayner, E., and Carter, W. Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique α and β subunits. J. Cell Biol., *105:* 1873–1884, 1987.
29. Wayner, E., Carter, W., Piotrowicz, R., and Kunicki, T. The functions of multiple extracellular matrix receptors in mediating cell adhesion to extra-

Downloaded from http://aacrjournals.org/cancerres/article-pdf/50/20/6757/2441486/cr0500206757.pdf by guest on 24 August 2022

cellular matrix: preparation of a monoclonal antibody to the fibronectin receptor that specifically inhibits adhesion to fibronectin and reacts with platelet glycoproteins Ic/IIa. J. Cell Biol., *107:* 1881–1801, 1988.
30. Hemler, M., Huang, C., and Schwarz, L. The VLA protein family. J. Biol. Chem., *262:* 3300–3309, 1987.
31. Hemler, M., Ware, C., and Strominger, J. Characterization of a novel differentiation antigen complex recognized by a monoclonal antibody (A1A5): unique activation-specific molecular forms on stimulated T cells. J. Immunol., *131:* 334- 340, 1983.
32. Sonnenberg, A., Modderman, P. W., and Hogervotst, F. Laminin receptor on platelets is the integrin VLA-6. Nature (London), *336:* 487–489, 1988.
33. Brass, L., Shatil, S., Kunicki, T., and Bennett, J. Effect of calcium on the stability of the platelet membrane glycoprotein IIb/IIIa complex. J. Biol. Chem., *260:* 7875–7881, 1985.
34. Cheresh, D., and Harper, J. Arg-Gly-Asp recognition by a cell adhesion receptor requires its 130 kDA a subunit. J. Biol. Chem., *262:* 1434–1437, 1987.
35. Albelda, S. M., Daise, M., Levine, E. M., and Buck C. A. Identification and characterization of cell-substratum adhesion receptors on cultured human endothelial cells. J. Clin. Invest., *83:* 1992–2002, 1989.
36. Elder, D., Guerry, D., Epstein, M., Zehngebot, C., Lusk, E., VanHorn, M., and Clark, W. Invasive malignant melanomas lacking competence for metastasis. Am. J. Dermatopathol., *6:* 55–62, 1984.
37. Wayner, E. A., Garcia-Pardo, A., Humphries, M. J., McDonald, J. A., and Carter, W. G. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-2) in plasma fibronectin. J. Cell Biol., *109:* 1321–1330, 1989.
38. Elices, M. J. Osborn, L., Takada, Y., Crouse, C., Luhowskyl, S., Hemler, M., and Lobb, R. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA/4/fibronectin binding site. Cell, *60:* 577–584, 1990.
39. Rice, E. G., and Bevilacqua, M. An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. Science (Washington DC), *246:* 1303-1306, 1989.
40. Kramer, R., and Marks, N. Identification of integrin collagen receptors on human melanoma cells. J. Biol. Chem., *264:* 4684–4688, 1989.
41. Mould, P. P., Wheldon, L. A., Komoriya, A., Wayner, E. A., Yamada, K. M., and Humphries, M. J. Affinity chromatographic isolation of the melanoma adhesion receptor for the IIICS region of fibronectin and its identification as the integrin $\alpha_4\beta_1$. J. Biol. Chem., *265:* 4020–4024, 1990.
42. Ramos D. M., Berston, E. D., and Kramer, R. H. Analysis of integrin receptors for laminin and type IV collagen on metastatic B16 melanoma cells. Cancer Res., *50:* 728–734, 1990.
43. Elder, D., Rodeck, U., Thurin, J., Cardillo, F., Clark, W., Stewart, R., and Herlyn, M. Antigenic profile of tumor progression stages in melanocytic nevi and melanomas. Cancer Res., *49:* 5091–5096, 1989.
44. Poste, G., and Greig, R. The experimental and clinical implications of cellular heterogeneity in malignant tumors. J. Cancer Res. Clin. Oncol., *106:* 159–170., 1983.
45. Nicolson, G. L. Generation of phenotypic diversity and progression in metastatic tumor cells. Cancer Metas. Rev., *3:* 25–42, 1984.
46. McGregor, B., McGregor, J. L., Weiss, L. M., Wood, G. S., Hu, C-H., Boukerche, H. and Warnke, R. A. Presence of cytoadhesins (IIb-IIIa-like glycoproteins) on human metastatic melanomas but not on benign melanocytes. Am. J. Clin. Pathol., *92:* 495–499, 1989.
47. Clark, W., Elder, D., Guerry, D., Braitman, L., Trock, B., Schultz, D., Synnestvedt, M., and Halpern, A. Model predicting survival in stage 1 melanoma based on tumor progression. J. Natl. Cancer Inst., *81:* 1893–1904, 1989.
48. Vogel, B., Tarone, G., Giancotti, F. G., Gailit, J., and Ruoslahti, E. A novel fibronectin receptor with an unexpected subunit composition (α_v/β_1). J. Biol. Chem., *265:* 5934–5937, 1990.
49. Krissansen, G. W., Elliott, M. J., Lucas, C. M., Stomski, F. C., Berndt, M. C., Cheresh, D. A., Lopez, A. F., and Burns, G. F. Identification of a novel integrin β subunit expressed on cultured monocytes (macrophages). J. Biol. Chem., *265:* 823–830, 1990.
50. Cheresh, D., Smith, J., Cooper, H., and Quaranta, V. A novel vitronectin receptor integrin ($\alpha_v\beta_x$) is responsible for distinct adhesive properties of carcinoma cells. Cell, *57:* 59–69, 1989.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/50/20/6757/2441486/cr0500206757.pdf by guest on 24 August 2022