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Role of α_V Integrins and Vitronectin in Human Melanoma Cell Growth

L.C. SANDERS, B. FELDING-HABERMANN, B.M. MUELLER, AND D.A. CHERESH
Department of Immunology, Scripps Research Institute, La Jolla, California 92037

The interaction of cells with the extracellular matrix (ECM) has been shown to facilitate many biological processes, such as cellular polarity, differentiation, proliferation, and migration (Hay 1981; Hynes 1981). A family of cell-surface receptors termed integrins play a fundamental role in promoting cell-matrix interactions as well as cell-cell interactions (Cheresh 1992; Hynes 1992). Consequently, these interactions can result in the transmission of signals from the extracellular environment to the intracellular compartment, providing a cellular mechanism that can convey various biological functions (Werb et al. 1989; Jaconi et al. 1991; Kornberg et al. 1991; Schwartz et al. 1991a,b; Leavesley et al. 1992).

Integrins are heterodimeric molecules composed of an α and a β subunit. Both subunits traverse the membrane and consist of large extracellular domains coupled to relatively small cytoplasmic tails. To date, 14 α and 8 β subunits have been described, making up at least 20 individual integrin receptors (Cheresh 1992; Hynes 1992). Depending on the α/β subunit pairing, the intact heterodimer can recognize one or multiple ligands present in the ECM or on the surface of opposing cells. In some cases, a given β subunit can associate with multiple α subunits. Alternatively, a specific α subunit can associate with a wide variety of β subunits, thus achieving diversity in the specificity and function of integrins. An example of the latter is the α_V integrin subunit, which can pair with multiple β subunits (β_1, β_3, β_5, β_6, β_8), each demonstrating differences in cell and/or tissue distribution and ligand specificity (Cheresh 1992). The most widely studied α_V integrins are $\alpha_V\beta_3$ and $\alpha_V\beta_5$, both of which mediate cell attachment to the ECM adhesive glycoprotein vitronectin (VN) (Cheresh et al. 1989; McLean et al. 1990; Ramaswamy and Hemler 1990; Smith et al. 1990; Wayner et al. 1991).

VN is a multifunctional adhesive glycoprotein known to promote the attachment and spreading of many different cell types in vitro. Cells can interact with VN either through its RGD domain, which involves integrins, or through its heparin-binding domain, which can bind proteoglycans on the cell surface (Reisfeld and Cheresh 1987; Tomasini and Mosher 1991). VN, like many other adhesive proteins, is produced in the liver and accumulates in the serum. Its regulatory role in the immune system and in fibrinolysis is well documented (Preissner 1989). VN not only exists in plasma, but has also been localized in the ECM of many different tissue types; these include human embryonic lung, fetal membrane, striated skeletal muscle, skin, and kidney (Hayman et al. 1983). Although the in vivo function of VN within the ECM is unclear, it has been suggested that it may be involved in cell adhesion and migration (Preissner 1989; Tomasini and Mosher 1991). Furthermore, increased VN deposits within skin ECM have been correlated with skin diseases such as discoid lupus erythematosus and dermatitis herpetiformis (Dahlback et al. 1989a,b). The observation that VN can be localized within human glioblastoma biopsy material suggests that VN may be involved in the progression of this highly lethal form of brain cancer (Gladson and Cheresh 1991). Recent immunohistological evidence indicates that VN accumulates in late-stage melanoma (C.L. Gladson and D.A. Cheresh, unpubl.). Interestingly, deposits of VN within the skin have been shown to increase with age, and additional evidence suggests that VN deposits may increase with exposure to the sun (Dahlback et al. 1989b). Both aging and sun exposure enhance the likelihood of contracting melanoma. Together, these observations implicate VN involvement in the progression of human melanoma.

It is not clear why more than one VN receptor exists or why multiple VN receptors are expressed by the same cell. However, it is tempting to suggest that each may send distinct signals to the cell, thereby potentiating separate biological functions in response to a VN substrate. Experimental evidence supports this contention. For example, two primary VN receptors, $\alpha_V\beta_3$ and $\alpha_V\beta_5$, contain identical α subunits with structurally distinct β subunits. Although β_3 and β_5 are 56% identical overall, their cytoplasmic tails are only 10–20% homologous. A structural motif unique to β_5 consists of a 10-amino-acid putative insert at the carboxyl terminus that has not been found on any other integrin β subunit (McLean et al. 1990; Ramaswamy and Hemler 1990). Although both $\alpha_V\beta_3$ and $\alpha_V\beta_5$ bind VN, recent evidence demonstrates that only $\alpha_V\beta_3$ is involved in cell spreading and is localized to focal contacts (Wayner et al. 1991). Cells containing only $\alpha_V\beta_5$ attached to VN but failed to spread, and $\alpha_V\beta_5$ was not detected in focal contacts by immunofluorescence (Wayner et al. 1991). Moreover, $\alpha_V\beta_3$ appears to promote migration of cells on VN, whereas $\alpha_V\beta_5$ does not (Leavesley et al. 1992). These data imply that the distinct cytoplasmic tails of β_3 and β_5 may differentially affect the biological responsiveness of a cell to a VN matrix.

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Integrin-mediated cellular adhesion mechanisms have been implicated in the tumorigenicity and invasive properties of transformed cells (Humphries et al. 1986; Plantefaber and Hynes 1989; Ruoslahti and Giancotti 1989; Felding-Habermann et al. 1992; Seftor et al. 1992). Both in vitro and in vivo experiments suggest that a change in integrin expression occurs during malignant transformation (Plantefaber and Hynes 1989; Ruoslahti and Giancotti 1989). For example, overexpression of the $\alpha_5\beta_1$ integrin (the fibronectin receptor) has been correlated with decreased tumorigenicity, whereas expression of $\alpha_V\beta_3$ has been associated with increased tumorigenicity and invasiveness of human melanoma cells (Albelda et al. 1990; Giancotti and Ruoslahti 1990; Felding-Habermann et al. 1992; Nip et al. 1992; Seftor et al. 1992). Integrin $\alpha_V\beta_3$ is commonly found on melanoma cultured cell lines (Cheresh and Spiro 1987). In fact, $\alpha_V\beta_3$ integrin is highly expressed on human melanoma biopsy material from metastatic and vertically invasive primary lesions; however, it is not expressed on normal melanocytes, nevi of noninvasive primary melanoma (Albelda et al. 1990). In vitro invasion assays using reconstituted basement membrane have demonstrated an increase in melanoma cell invasiveness when soluble VN or antibodies to $\alpha_V\beta_3$ were present (Seftor et al. 1992). These observations suggest not only that $\alpha_V\beta_3$ is a marker of invasive melanoma, but also that it may play an important role in the malignant behavior of this neoplasm.

M21 cells, a human melanoma cell line derived from a metastatic lesion, express both $\alpha_V\beta_3$ and $\alpha_V\beta_5$. To establish the biological role of these receptors, a stable variant cell line lacking α_V mRNA was selected (M21-L) (Cheresh and Spiro 1987). Consequently, the M21-L cells fail to express $\alpha_V\beta_3$ and $\alpha_V\beta_5$ on their cell surface and do not attach to VN, fibrinogen, or von Willebrand factor (Cheresh and Spiro 1987). Therefore, the M21 and M21-L cells provide a model system to address the role of α_V integrins in the malignant phenotype of human melanoma. In this study, we assessed the role of α_V integrins and VN in the tumorigenicity of human melanoma cells in vivo. Specifically, we examined the growth properties of both M21 and M21-L cells for their ability to form tumors in athymic nude mice. Results presented here indicate that melanoma tumorigenicity depends on the expression of α_V integrins and the availability of VN in situ.

EXPERIMENTAL PROCEDURES

Cell propagation. M21 human melanoma cells and variants (M21-L, M21-L4, M21-L12) were propagated in culture using RPMI 1640 containing 10% fetal bovine serum as described previously (Cheresh and Spiro 1987). Cells grown in vivo were washed and resuspended in 0.1 phosphate-buffered saline (PBS) (pH 7.2), then injected subcutaneously into the flanks of 6-week-old female nude mice (Felding-Habermann et al. 1992). Coinjection of cells with VN and collagen occurred as described above with the addition of 500 μg/injection of protein.

Transfection of M21-L cells with α_V. M21-L cells were transfected with the plasmid pcDNA I/NEO (Invitrogen) containing α_V cDNA as described previously (Felding-Habermann et al. 1992). In brief, transfected cells were cultured in growth medium containing 500 μg/ml Geneticin (Sigma). From a panel of transfected subpopulations, cells expressing wild-type levels of α_V integrins (M21-L4) and nonexpressing control cells (M21-L12) were selected by repeated fluorescence-activated cell sorting using anti-α_V MAb LM142.

Cell adhesion assay. Cell adhesion experiments were performed as described previously (Wayner et al. 1991). In brief, ^{51}Cr-labeled cells were suspended in Hank's balanced salt solution containing 1% bovine serum albumin (BSA), 1 mM calcium, 1 mM magnesium, and 0.5 mM manganese. Cells were seeded in 48-well non-tissue-culture plastic microtiter plates, precoated with individual adhesive ligands (10 μg/ml). After adhesion, unbound cells were washed away, and adherent cells were harvested and counted. BSA-coated wells were used as a control.

Cell proliferation assay. M21 cells and variants were suspended in endothelial cell basal medium (EBM) (Clonetics Corporation) containing no serum or growth factors. Cells were seeded in 96-well non-tissue-culture plastic microtiter plates, precoated with adhesive ligands (10 μg/ml). At various time points after seeding, the increase in viable cell number was enumerated using a blue formazan colorimetric assay (Promega). BSA-coated wells were used as a control.

RESULTS AND DISCUSSION

To address the potential role of α_V integrins and VN in the malignant phenotype of human melanoma cells, a cell model system has been developed. Human melanoma cells (M21 cells) were selected by fluorescence-activated cell sorting for the lack of α_V expression. The resulting cell population (M21-L cells) fails to express α_V integrins (Fig. 1). Previous studies have demonstrated that M21-L cells fail to produce mRNA encoding α_V (Cheresh and Spiro 1987). These cells represent a stable population, since they have been passaged for several years and remain α_V negative. M21-L cells lacking α_V have lost expression of both $\alpha_V\beta_3$ and $\alpha_V\beta_5$ but maintain equivalent levels of β_1 integrins (Fig. 1). To examine whether the loss of α_V results in an altered adhesive phenotype, M21 or M21-L cells were allowed to attach to VN, fibronectin, types I and IV collagen, laminin, fibrinogen, and von Willebrand factor. As shown in Figure 2, M21-L cells readily attach to fibronectin, both collagens, and laminin, consistent with their expression of β_1 integrins. However, M21-L cells failed to attach to VN, fibrinogen, and von Willebrand factor, demonstrating their lack of α_V integrin expression (Fig. 2).

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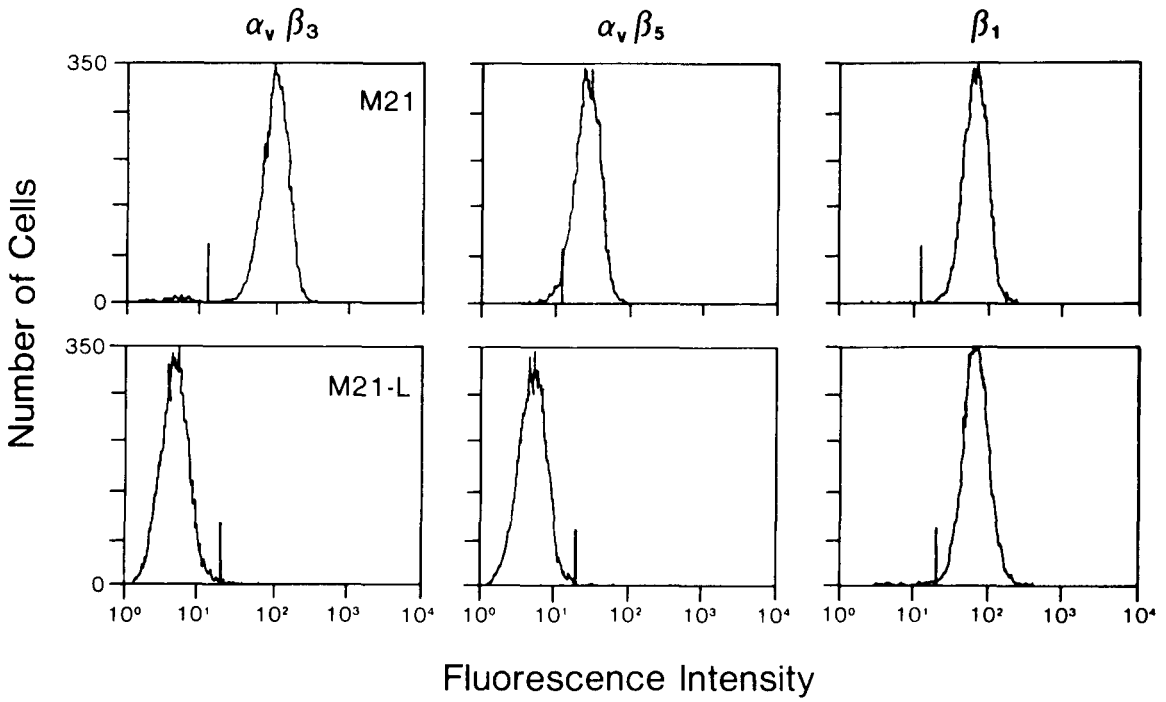

Figure 1. α_V integrin expression on M21 and M21-L cells. Flow cytometric analysis demonstrates differential expression of α_V integrins on M21 cells and M21-L cells. M21 cells express both $\alpha_V\beta_3$ and $\alpha_V\beta_5$, whereas M21-L cells express neither. M21 and M21-L express equivalent amounts of β_1 integrins.

To examine the growth properties of these cell lines, M21 and M21-L cells were injected subcutaneously into athymic nude mice. M21-L cells show a dramatic reduction in tumorigenicity compared to the M21 parental cell population (Fig. 3A). In vitro, however, these cells grow at the same rate, indicating that their growth properties are not intrinsically different (Fig. 3B). Moreover, when M21 or M21-L cells were cultured from their respective tumors, the cells maintained their respective α_V expression (data not shown). These data indicate that the reduction in tumor growth of M21-L cells is not due to in vivo selection of α_V-expressing cells.

To demonstrate a direct role for α_V integrins in human melanoma tumor growth, M21-L (α_V^-) cells were transfected with a cDNA encoding α_V (M21-L4 cells). This resulted in wild-type levels of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ expression on the cell surface (Fig. 4). These cells regained the capacity to attach to VN, and tumorigenicity was fully restored (Figs. 5 and 6). A control cell population, M21-L12 cells (mock transfectants; α_V^-) failed to form significant tumors and were incapable of binding VN, behaving as the M21-L cells (Figs. 5 and 6). These results demonstrate that integrin α_V gene expression and the resulting adhesive phenotype are directly involved in the in vivo proliferation of human

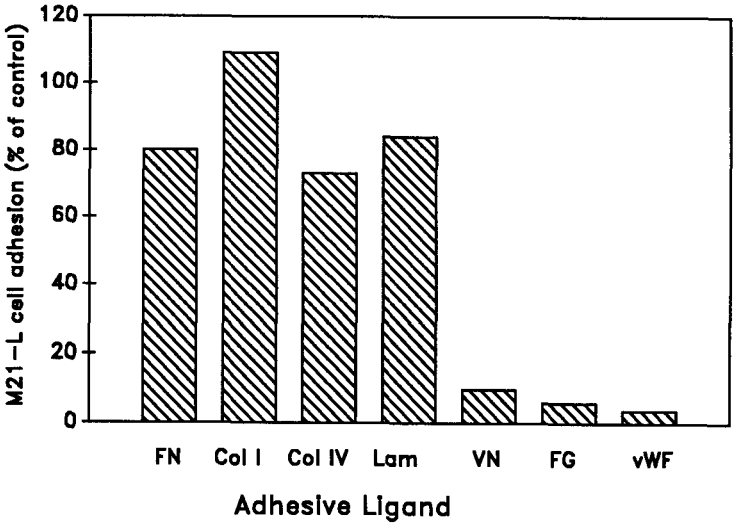

Figure 2. Adhesion of M21-L cells to various ligands. Attachment to various ligands demonstrates the different adhesive properties of M21-L (α_V^-) cells compared to M21 (α_V^+) cells. M21-L cell adhesion is plotted as percent of M21 cell adhesion. M21-L cells maintain their ability to adhere to fibronectin (FN), collagen type 1 (Col I), collagen type IV (Col IV), and laminin (Lam), presumably due to their equivalent β_1 expression. However, M21-L cells do not attach to VN, fibrinogen (FG), or von Willebrand factor (vWF), due to their loss of α_V integrin expression.

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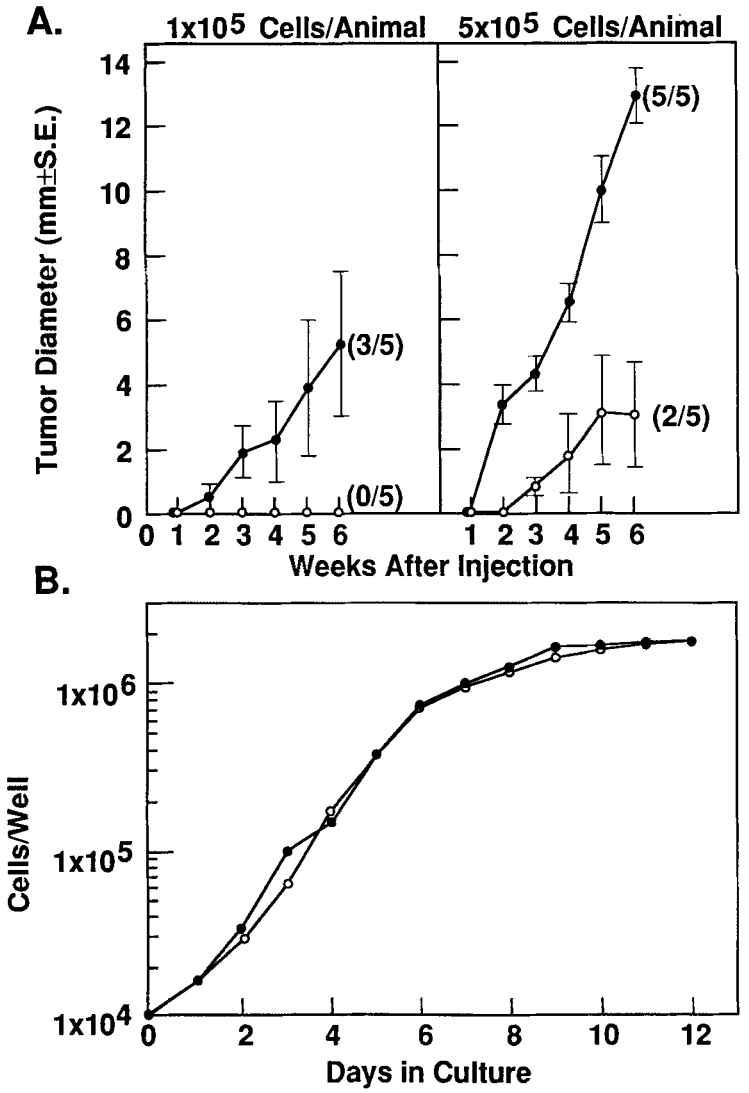

Figure 3. In vivo and in vitro growth properties of M21 and M21-L cells. (*A*) M21 human melanoma cells (●) or M21-L cells deficient in mRNA for α_V integrin (○) show different growth properties in vivo when injected subcutaneously into nude mice. (*B*) In vitro growth of M21 (●) and M21-L cells (○) was indistinguishable in complete growth medium containing 10% serum. (Reprinted, with permission, from Felding-Habermann et al. 1992 [copyright held by American Society for Clinical Investigation].)

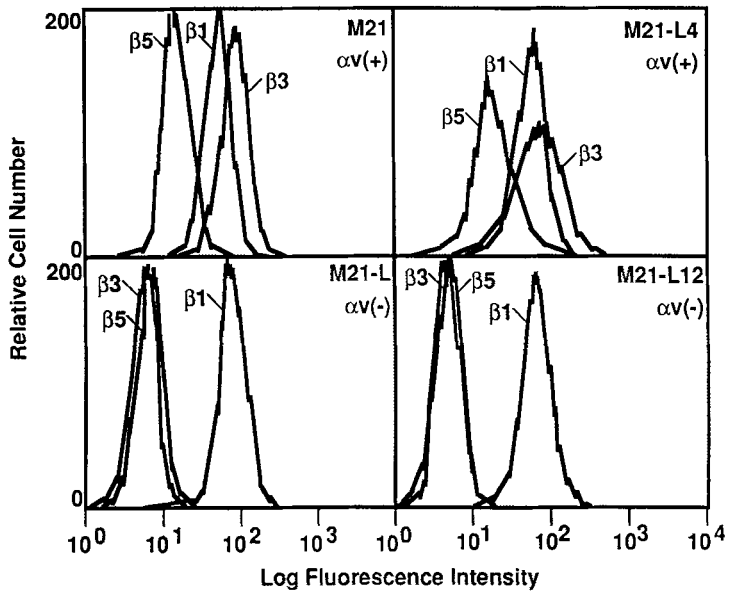

Figure 4. α_V and β_1 integrin expression on M21 cell variants. Flow cytometric analysis demonstrates differential expression of α_V integrins on M21 cell variants. M21 (wild type) and M21-L4 (transfected with cDNA for α_V) cells express $\alpha_V\beta_3$ and $\alpha_V\beta_5$; in contrast, M21-L (stable variant lacking α_V integrins) and M21-L12 (mock transfectant) cells do not. (Reprinted, with permission, from Felding-Habermann et al. 1992 [copyright held by American Society for Clinical Investigation].)

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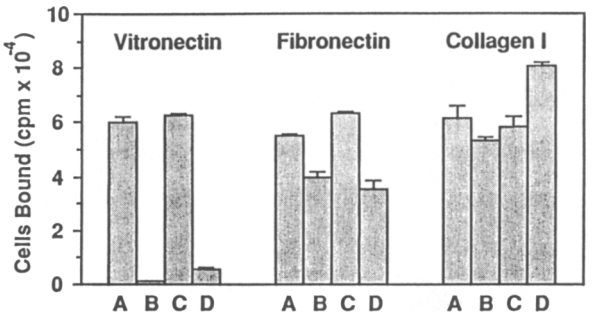

Figure 5. Adhesive properties of M21 cell variants. Attachment to immobilized ligands demonstrates the different adhesive properties of M21 cell variants. M21 and M21-L4 (α_V^+) cells adhere to VN, whereas M21-L and M21-L12 (α_V^-) cells do not. All four cell types attach to fibronectin and collagen consistent with their equivalent expression of β_1 integrins. (*A*) M21 cells; (*B*) M21-L cells; (*C*) M21-L4 cells, and (*D*) M21-L12 cells. (Reprinted, with permission from Felding-Habermann et al. 1992 [copyright held by American Society for Clinical Investigation].)

melanoma cells. In addition, these findings suggest that a ligand for α_V integrins such as VN may be capable of promoting the tumorigenicity of human melanoma cells.

In contrast to their in vivo growth differences, the M21 cells and adhesive variants proliferate identically in vitro, in the presence of serum (Fig. 3). However, in preliminary experiments when serum and exogenous growth factors are removed, these cells demonstrate anchorage-dependent proliferation (Fig. 7). Specifically, the α_V-expressing M21 and M21-L4 cells (positive α_V transfectants) proliferate in response to immobilized VN, demonstrating a mean doubling time of 96 hours. In contrast, M21-L and M21-L12 cells that lack α_V integrins fail to respond to immobilized VN (Fig. 7). However, all four cell types proliferate in response to collagen and fibronectin, which is likely due to their equivalent expression of β_1 integrins (Felding-Habermann et al. 1992). To our knowledge, this is the first demonstration of anchorage-dependent tumor cell proliferation. By establishing growth conditions in vitro that require α_V integrins, thereby mimicking the in vivo growth experiments, we are in a position to examine the role of α_V integrins in the proliferation of human melanoma cells. Our preliminary data suggest that VN, in vitro, can promote a proliferative signal to human melanoma cells by interacting with an α_V integrin. Further evidence supports a role for VN in this process in vivo as well. Specifically, when VN is coinjected with M21 cells subcutaneously into nude mice, a fourfold increase in proliferation was observed over cells injected without VN ($p < 0.05$) (Figs. 8 and 9). This increased proliferation was not observed when M21-L cells (α_V^-) were coinjected with VN, indicating that this response was dependent on α_V integrins (data not shown). Yet, both α_V^+ cells (M21 and M21-L4) and α_V^- cells (M21-L12) show increased tumorigenicity when coinjected with collagen, which is likely due to equivalent levels of β_1 integrins (data not shown). These observations and the fact that VN can be localized within the parenchyma of human melanoma biopsy material (C.L. Gladson and D.A. Cheresh, unpubl.) suggest a role for VN and α_V integrins in the malignant phenotype of human melanoma.

It is widely accepted that tumor cell-matrix interactions facilitate the progression of cancer, and several studies support the role of tumor cell-surface integrins in this process (for review, see Cheresh 1992). The role α_V integrins play in tumor cell proliferation and invasion have only recently been explored. For example, RGD-containing peptides, known to block the function of $\alpha_V\beta_3$ integrin, have been shown to inhibit melanoma tumor invasion in vitro and experimental metastases in a murine melanoma model system (Humphries et al. 1986; Gehlson et al. 1988). Similarly, an antibody to the β_3 subunit has been reported to inhibit growth of human melanoma cells in nude mice (Boukerche et al. 1989). In vitro invasion assays have shown melanoma cell invasiveness increases in the presence of soluble VN or antibodies to $\alpha_V\beta_3$ (Seftor et al. 1992). Integrin $\alpha_V\beta_3$ is commonly found on melanoma cell lines and is

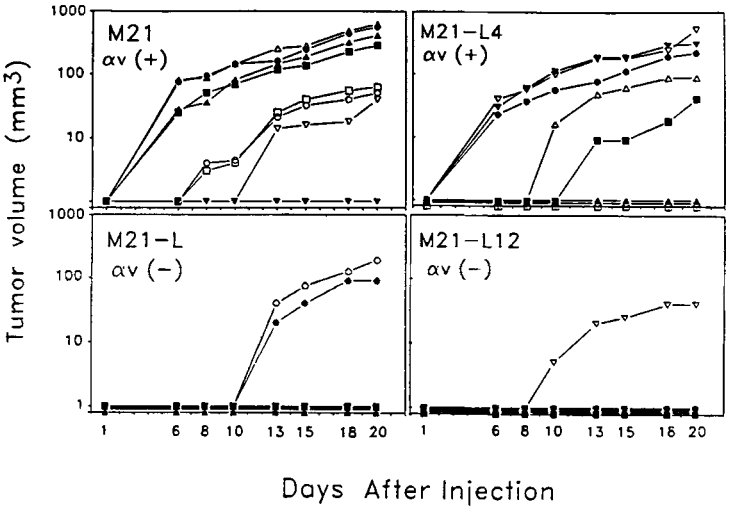

Figure 6. Tumorigenicity of M21 cell variants. M21 cell variants (1×10^6) were injected subcutaneously into the flanks of female nude mice. Tumors were allowed to proliferate for 20 days and the volume of each tumor was calculated ($w^2 \times 1/2$). M21 and M21-L4 (α_V^+) cells were significantly more tumorigenic than their α_V^- counterparts ($p < 0.01$ as determined by the Wilcoxon rank sum test). (Reprinted, with permission, from Felding-Habermann et al. 1992 [copyright held by American Society for Clinical Investigation].)

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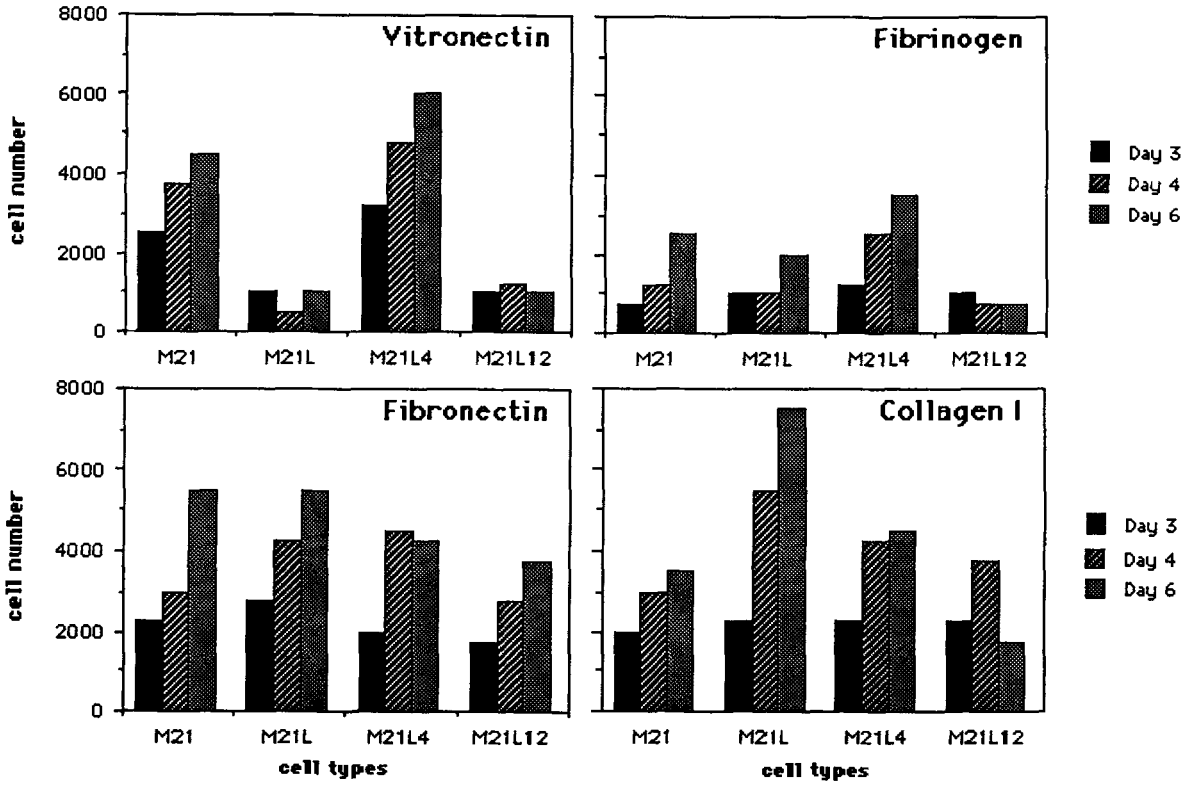

Figure 7. In vitro integrin-mediated anchorage-enhanced proliferation of human melanoma cells. M21 cell variants display different growth properties in response to immobilized adhesive ligands when grown in vitro, in the absence of serum and exogenous growth factors. M21 and M21-L4 (α_V^+) cells proliferate when grown in the presence of VN, whereas M21-L and M21-L12 (α_V^-) cells do not. Fibrinogen had a slight stimulative effect on the α_V^+ cells and no effect on the α_V^- cells. All four cell types showed proliferation in response to fibronectin and collagen, likely due to their identical β_1 integrin profile.

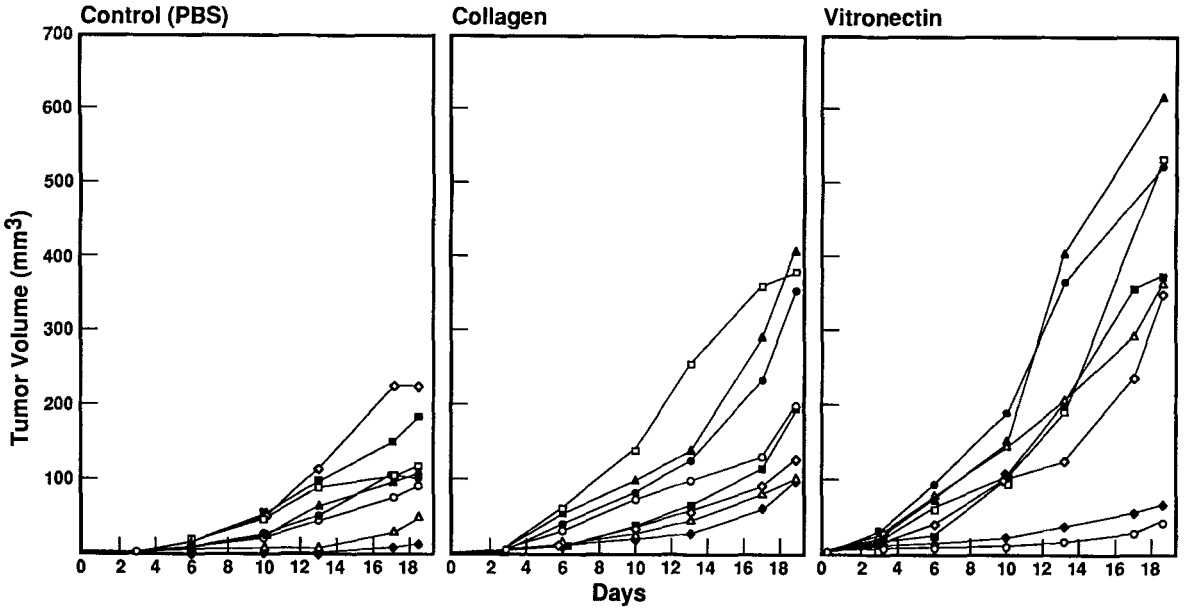

Figure 8. Enhancement of human melanoma tumorigenicity by extracellular matrix proteins. M21 cells (5×10^5) show enhanced tumorigenicity when subcutaneously coinjected with extracellular matrix molecules (500 μg/injection) into the flanks of nude mice. M21 (α_V^+) cells showed a three- to fourfold increase in tumor volumes when coinjected with VN and a twofold increase when coinjected with collagen, compared to cells injected with PBS alone.

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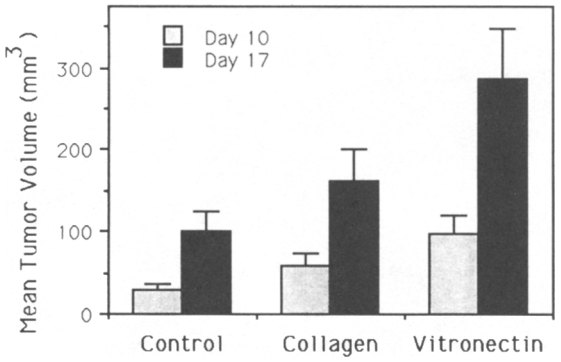

Figure 9. Extracellular matrix proteins enhance tumorigenicity of M21 cells. M21 tumor volumes were compared 10 and 17 days postinjection. M21 tumor volumes derived from cells coinjected with VN, when compared to tumor volumes obtained from cells injected with PBS alone, showed a significant difference at $p < 0.05$ (determined by the Wilcoxon rank sum test).

preferentially expressed on melanoma tissues, including vertically invasive primary and metastatic lesions, yet is absent on normal melanocytes, nevi, and horizontal primary melanoma lesions (Cheresh and Spiro 1987; Albelda et al. 1990). Metastatic melanoma, like many tumors, initiates at a primary site followed by cellular proliferation, and this can lead to invasion and metastasis. Our results indicate that specific growth conditions within the in vivo microenvironment favor cells that express α_V integrin(s). Consequently, it would appear that the expression of $\alpha_V\beta_3$ and/or $\alpha_V\beta_5$ is directly involved in the proliferative response of human melanoma in vivo. The mechanism by which this occurs is unclear; however, our preliminary results suggest the involvement of the extracellular adhesive ligand VN. Both α_V^+ cells (M21, M21-L4) and α_V^- cells (M21-L and M21-L12) grow at the same rate under typical tissue culture growth conditions — conditions that contain serum and growth factor. It is only when these cells are grown in non-serum conditions and in the presence of immobilized VN that they mimic the observed in vivo growth and thus demonstrate their anchorage dependence. These data strongly suggest that human melanoma cells have a distinct proliferative advantage in the presence of an adhesive ligand (i.e., VN) both in vivo and in vitro.

Nontransformed cells proliferate in an anchorage-dependent manner requiring adhesion to a matrix and stimulation by growth factors (End and Engel 1991). These extracellular signals stimulate an intracellular cascade of events leading to a proliferative response. A model is depicted in Figure 10 representing how these extracellular ligand-binding events may differ between normal and transformed cells. In this case, a normal cell requires signals from both growth factors and ECM molecules (anchorage-dependent growth); this synergy provides competency for normal cell division. However, a transformed cell is capable of responding to a signal potentiated by a growth factor (anchorage-independent growth) or an ECM molecule(s) (anchorage-dependent growth). This model may explain our observation that in the absence of serum and/or growth factors, binding of melanoma cells to VN through an α_V integrin stimulates proliferation. Consequently, VN acts as a mitogen for these cells. These observations provide evidence that the integrin-mediated cellular adhesion mechanism plays an important role in tumor cell proliferation.

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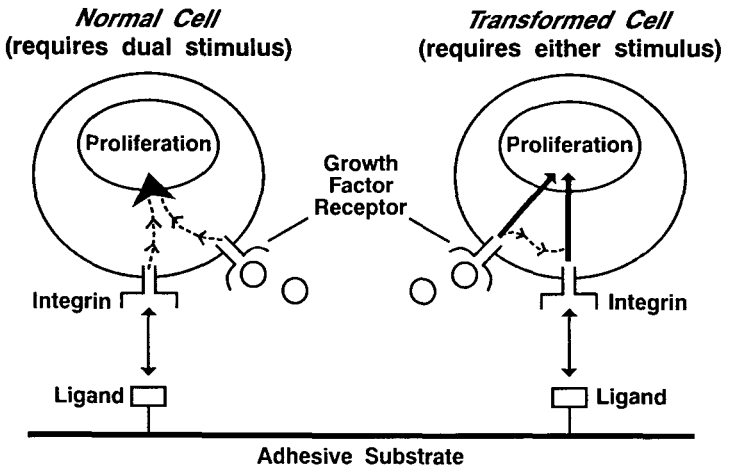

Figure 10. Cellular proliferation of normal versus transformed cells. A cellular proliferation model with emphasis on the extracellular signals required for cell proliferation. A normal cell requires stimulus from growth factors and ECM molecules, whereas a transformed cell requires stimulus from either.

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