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Role of integrins in melanocyte attachment and dendricity

Masahiro Hara, Mina Yaar, Aimin Tang, Mark S. Eller, Wende Reenstra and Barbara A. Gilchrest*

Boston University School of Medicine, Department of Dermatology, 80 E. Concord Street, Boston, MA 02118-2394, USA

*Author for correspondence

SUMMARY

Integrins are a family of proteins known to mediate attachment of cells to extracellular matrix materials. The substratum specificity and cation dependence of specific integrin heterodimers have been extensively characterized, and to a lesser degree specialized roles in cell attachment versus dendricity have been defined in some cell types. In the past decade, melanocyte attachment rate and morphology have been found to have strong substratum dependence, suggesting a major role for integrins in these processes. In order to investigate this aspect of pigment cell biology, human newborn melanocytes were subjected to flow cytometry analysis and plated on a variety of substrata under conditions known to promote or block the binding of specific integrin pairs. Melanocyte attachment to laminin and type IV collagen was promoted by Mg^{2+} and Mn^{2+} but not by Ca^{2+}, in the range of concentrations examined. However, dendrite outgrowth from melanocytes already attached on laminin or type IV collagen was promoted by Ca^{2+} to a far greater degree than by Mg^{2+}, and Mn^{2+} had no effect on dendrite outgrowth. Flow cytometry analysis revealed that melanocytes expressed β_1, α_2, α_3, α_5, α_6 and α_v integrin subunits as well as the $\alpha_v\beta_3$ heterodimer. The influence of substratum on the profile of integrin expression was minimal, but α_6 and β_1 integrins were observed by confocal microscopy to be expressed over the entire cell surface, while α_2, α_5 and $\alpha_V\beta_3$ integrins localized along dendritic processes or at their tips. In accordance with the implications of these distribution patterns, anti-β_1 and anti-α_6 integrin monoclonal antibodies blocked melanocyte attachment to laminin, while anti-α_2, anti-α_5 and anti-$\alpha_v\beta_3$ inhibited dendrite outgrowth but did not block substratum attachment on either laminin or type IV collagen. On the basis of these data and the known characteristics of integrin molecules, we conclude that melanocyte attachment to laminin is mediated primarily by $\alpha_6\beta_1$ integrin in a Ca^{2+}-independent, Mg^{2+}- and/or Mn^{2+}-dependent manner, while dendrite outgrowth on laminin and type IV collagen requires extracellular Ca^{2+} and is mediated by $\alpha_v\beta_3$ as well as α_2 and α_5 integrins.

Key words: integrin, melanocyte, laminin, type IV collagen, attachment, dendricity

INTRODUCTION

Extracellular matrix (ECM) molecules have important regulatory roles for several cellular activities including migration (Aznavoorian et al., 1990; Calof and Lander, 1991), morphology (Tomasek et al., 1982), gene induction (Blum et al., 1987; DiPersio et al., 1991; Streuli et al., 1991), growth and differentiation (Adams and Watt, 1989; Watt et al., 1993; Woodley et al., 1990). In particular they are known to affect the dendricity of neural crest cells (reviewed by Hynes and Lander, 1992). Laminin (LM) is known to promote neurite outgrowth in a wide variety of neurons (Lander et al., 1985; Manthorpe et al., 1882; Rogers et al., 1983) as well as in PC12 cells (Tomaselli et al., 1990; Turner et al., 1987). Fibronectin (FN) and, to a lesser extent, type I and type IV collagens (CIV) as well as vitronectin (VN) and thrombospondin also influence neurite outgrowth of a variety of avian and mammalian neurons (Akers et al., 1981; Bozyczko and Horwitz, 1986; Neugebauer et al., 1991; O'Shea et al., 1991). However, the exact mechanisms by which ECMs promote neurite outgrowth in neural crest-derived cells is not well understood.

Cells interact with ECM through a family of adhesion molecules called integrins (reviewed by Albelda and Buck, 1990; Hynes, 1992; Ruoslahti, 1991). Integrins are transmembrane heterodimeric glycoprotein receptors present on the surface of many types of cells. Based on amino acid homology, integrins are divided into two major subgroups, α and β. One α and one β subunit are dimerized to form a particular integrin pair in the presence of extracellular divalent cations. At least 8 β and 13 α subunits are known, and 19 different functional combinations have been identified. Ligands recognized by integrins include not only ECM components, but also serum proteins like fibrinogen, von Willebrand factor, complement fragment C3bi and cell surface proteins of the immunoglobulin superfamily, including ICAMs and VCAM-1 (reviewed by Albelda and Buck, 1990; Hynes, 1992; Ruoslahti, 1991).

β_1 and β_3 integrin families, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_1$ and $\alpha_v\beta_3$, are known to interact with ligands present in the basement membrane and in the interstitial matrix and are thus candidates for mediating epithelial or endothelial cell interactions with ECM (reviewed by Albelda and Buck, 1990; Hynes, 1992; Ruoslahti, 1991). A given integrin heterodimer pair may recognize multiple ECM ligands and, conversely, several different integrins may recognize the

same ligand. Most cells express several integrins on their surface. However, which integrin pair is expressed and its distribution on the cell surface appear to depend on a combination of extracellular and intracellular signals, which to date are poorly understood (reviewed by Albelda and Buck, 1990; Hynes, 1992; Ruoslahti, 1991).

Integrins require divalent cations not only for dimerization but also for ligand binding (D'Souza et al., 1991; Gulino et al., 1992; Loftus et al., 1990; Masumoto and Hemler, 1993). Cations may act to increase the affinity and specificity of integrin heterodimers for their ligand or they may compete with each other to decrease ligand affinity. Among β_1 and β_3 integrin subfamilies, $\alpha_5\beta_1$ integrin can bind its ligand in the presence of Ca^{2+} as well as Mg^{2+} and/or Mn^{2+} (Gailit and Ruoslahti, 1988; Kirchhofer et al., 1991). $\alpha_v\beta_3$ mediates endothelial cell migration on vitronectin and this is totally extracellular Ca^{2+}-dependent (Leavesley et al., 1993). Other heterodimers such as $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_6\beta_1$ and $\alpha_v\beta_1$ require Mg^{2+} and/or Mn^{2+} to bind their ligands; and Ca^{2+} conversely inhibits their ligand binding (Elices and Hemler, 1989; Forsberg et al., 1990; Hall et al., 1990; Lallier and Bronner-Fraser, 1992; Sonnenberg et al., 1988, 1990; Staatz et al., 1989). $\alpha_3\beta_1$ heterodimer requires Ca^{2+} to bind LM and collagen but binds FN in the absence of Ca^{2+} (Elices et al., 1991).

Epidermal melanocytes are neural crest-derived cells that migrate to the epidermis early during embryonic development and then reside in the epidermal basal layer, transferring melanin granules to surrounding keratinocytes through dendritic processes that extend into the suprabasilar layers (Yaar and Gilchrest, 1991). Previous work has shown that attachment rate and morphology of cultured human melanocytes strongly depend on their substratum (Gilchrest et al., 1985; Ranson et al., 1988) and that melanocytes express several integrins (Morelli et al., 1993; Scott et al., 1992; Zambruno et al., 1993). We now report that melanocyte attachment to LM and CIV is mediated by Mg^{2+}/Mn^{2+}-dependent Ca^{2+}-independent integrins, but that dendrite formation on the same substrata require extracellular Ca^{2+} and is mediated by Ca^{2+}-dependent integrins in addition to Mg^{2+}/Mn^{2+}-dependent integrins. Moreover, integrins that are involved in dendrite formation are located preferentially on melanocyte dendrites, while integrins involved in melanocyte attachment are present primarily on melanocyte cell body. Our data suggest a major role for integrin heterodimers in melanocyte attachment and dendricity, and are consistent with their known topological situation on a LM- and CIV-containing epidermal basement membrane in a low Ca^{2+} environment, with extension of dendrites into the higher Ca^{2+} environment of the suprabasilar epidermis.

MATERIALS AND METHODS

Extracellular matrix molecules and monoclonal antibodies

Human fibronectin (FN), human vitronectin (VN), human type IV collagen (CIV) and mouse laminin (LM) were purchased from Becton Dickinson (Bedford, MA). Bovine serum albumin (BSA) and poly-L-lysine (Poly-L) were obtained from Sigma Chemical Company (St Louis, MO). Mouse mAb P1E6 (anti-human α_2 integrin, IgG_1), mouse mAb P1B5 (anti-human α_3 integrin, IgG_1), mouse monoclonal antibody (mAb) P1D6 (anti-human α_5 integrin, IgG_3), mouse mAb VNR147 (anti-human α_v integrin, IgG_1), mouse mAb P4C10 (anti-human β_1 integrin, IgG_1) and Streptavidin-RED^{613} were purchased from Gibco BRL Life Technologies, Inc. (Grand Island, NY). Rat mAb CLD-701 (anti-human α_6 integrin, IgG_{2b}) and mouse mAb LM609 (anti-human $\alpha_v\beta_3$ integrin, IgG) were purchased from CHEMICON International, Inc. (Temecula, CA). Chromatographically purified mouse and rat control immunoglobulins (IgGs) were obtained from Cappel (Organon Teknika Corporation, West Chester, PA) and Sigma, respectively. FITC-conjugated goat anti-mouse and anti-rat IgG were obtained from Sigma. Biotin-conjugated affinity-purified $F(ab')_2$ fragments of goat anti-rat IgG (human and mouse IgG absorbed) was purchased from Tago, Inc. (Burlingame, CA) and biotinylated $F(ab')_2$ fragment of affinity-isolated rabbit anti-mouse IgG was obtained from DAKO Co. (Carpinteria, CA). Antibody concentrations were assayed by indirect ELISA.

Melanocyte culture and medium

Neonatal foreskins obtained within 24 hours of elective circumcision were used to culture human melanocytes as previously described (Gilchrest et al., 1984; Park et al., 1993). In brief, the epidermis was separated from the dermis after overnight incubation in 0.25% trypsin at 4°C. Primary cultures were then established in Medium 199 (Gibco BRL, Grand Island, NY) supplemented with 10 ng/ml EGF (Bethesda Research Laboratories, Gaithersburg, MD), 10^{-9} M triiodothyronine (Sigma), 10 μg/ml transferrin (Sigma), 10 μg/ml insulin (Sigma), 1.4×10^{-6} M hydrocortisone (Calbiochem-Behring Corp., La Jolla, CA), 10^{-9} M cholera toxin (List Biological, Campbell, CA), 10 ng/ml basic fibroblast growth factor (Amgen, Thousand Oaks, CA), and 5-10% fetal bovine serum (FBS). All post-primary cultures were maintained in a low calcium (0.03 mM) version of this defined melanocyte growth medium known to selectively support melanocyte growth (Naeyaert et al., 1991).

For cell attachment studies a HEPES-buffered minimal attachment medium (MAM) modified from Klebe et al. (1977) was used to ensure solubility of divalent cations (6.78 g/liter NaCl, 0.4 g/liter KCl, 1 g/liter D-glucose, 2.38 g/liter HEPES, 0.01 g/l Phenol Red, pH 7.4). All other experiments employed a specially formulated HEPES-buffered Medium 199 (Gibco) lacking cations but supplemented with all other growth factors and hormones, as listed in the previous paragraph, as well as with 0.1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$ or combinations of these cations; concentrations were determined in preliminary studies to be the lowest sufficient for optimal melanocyte attachment.

Substratum coating

A 96-well Falcon® plate (Becton Dickinson & Company, NJ) or 8-chamber glass slides (Nunc Inc., Naperville, IL) were coated overnight with 3 μg/cm^2 of FN, LM, CIV, VN or 5 μg/ml of Poly-L in HEPES buffered saline (HBS) at 4°C. After rinsing with HBS, wells or chamber glass slides were incubated with 3% BSA in HBS for 1 hour at 37°C to block nonspecific binding sites and then rinsed two times with HBS.

Cell attachment assay

Cell attachment assays were carried out in a 96-well plates using a modification of the method of Turner et al. (1987). Confluent melanocyte cultures were radiolabelled with 50 μCi/ml of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) for 2-3 hours in methionine-free medium. This incubation time was determined in preliminary studies. After washing with 5 mM EDTA to remove free [^{35}S]methionine and divalent cations, cells were trypsinized, resuspended at 4×10^6 cells/ml and plated on substratum-coated wells in the various cation media. Melanocytes were then allowed to attach to the substratum for 90 minutes. Unattached cells were removed by two MAM washes. Attached cells were lysed with 0.1% SDS in PBS for 30 minutes, transferred to scintillation vials and counted in a beta counter. Assays were performed in duplicate and at

least two experiments were performed. The percentage of attached cells was calculated as [(cpm of radiolabelled cells attached to wells)/(cpm of total applied radiolabelled cells)]×100.

In the attachment inhibition experiments melanocytes were plated in MAM supplemented with 1 mM $CaCl_2$, $MgCl_2$ and 0.1 mM $MnCl_2$ to LM-coated 96-well plates in the presence of 50 μg/ml of primary antibodies. Non-adherent melanocytes were removed after 90 minutes by washing with MAM and attached cells were fixed with 2.5% glutaraldehyde followed by staining with Crystal Violet. The attached cells were then dissolved in 1% SDS and the relative number of cells in each well was evaluated by measuring the absorbance at 550 nm in an ELISA reader. This method, widely used for studies of integrin binding (Hall et al., 1990; Tomaselli et al., 1990), gave far more reliable results in the presence of blocking antibodies than the above method used initially for our experiments.

Evaluation of dendrite outgrowth on ECMs

Melanocytes (1×10^3 to $5\times10^{3)}$ were plated on ECM-coated 8-chamber glass slides with HEPES-buffered defined Medium 199 supplemented with 0.1 mM $MnCl_2$, 1 mM $MgCl_2$ or 1 mM $CaCl_2$ alone or in combination and were incubated for 1, 2, 4, 8 or 24 hours. Pictures (×100 magnification) were taken through a phase-contrast microscope (Nikon) and used to assess melanocyte morphology. Cells with more than one narrow process exceeding the cell body diameter in length were classified as dendritic (Heidemann et al., 1985). At least 300 cells were counted for each condition.

Melanocyte attachment and dendricity blocking experiments by mAbs against each integrin were performed in HEPES-buffered M199 medium supplemented with 0.1 mM $MnCl_2$, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ as well as growth factors. Concentrations of 5, 10 or 50 μg/ml of mAbs against β_1, α_2, α_3, α_5, α_6 and α_v integrin subunits as well as $\alpha_v\beta_3$ heterodimer, all reported to inhibit integrin function in other cell types (Gailit and Ruoslahti, 1988; Kirchhofer et al., 1991; Staatz et al., 1989; Sonnenberg et al., 1990; Elices et al., 1991), were added to duplicate chambers immediately after plating the melanocytes. Micrographs were taken 2 hours after plating.

Immunofluorescent staining and confocal laser scanning microscopy

Six hours after plating on LM- and CIV-coated glass slides, melanocytes were fixed with 4% paraformaldehyde for 20 minutes at 4°C, then incubated with 2% normal goat serum for 10 minutes to block nonspecific binding sites for secondary antibodies. Cells were then incubated overnight at 4°C with mAbs against β_1, α_2, α_3, α_5, α_6 and $\alpha_v\beta_3$ at concentrations of 20-30 μg/ml. After washing with PBS, cultures were incubated with FITC-conjugated goat anti-mouse IgG or goat anti-rat IgG (25 μg/ml or 10 μg/ml, respectively) for 1 hour at room temperature. Then the specimens were rinsed three times with PBS and mounted with anti-fade reagent (Molecular Probes, Inc., Eugene, OR) for observation.

Specimens were analyzed with a Leica upright confocal laser scanning microscope (CLSM) that is equipped with an argon ion laser with an output power of 2-50 mW. The continuously variable detection pinhole was set at the minimal size for optimal signal. The smaller pinhole (aperture) allowed a narrower optical section and less background (Pawley, 1990; Svoboda, 1992; Trinkaus-Randall et al., 1993). The melanocytes plated on glass slides were scanned at first in the *xy* plane from top to bottom of the cells. Each image of the optical section was taken at intervals from 0.5 to 1 μm in the 512×512 pixel format. Pictures shown in Fig. 9 were taken at the ECM-cell membrane interface, to observe the localization of integrins at binding sites.

Flow cytometry

Seven hours after plating on LM, CIV or Poly-L, melanocytes were detached with 5 mM EDTA and suspended in staining buffer composed of PBS supplemented with 5% (v/v) calf serum and 0.02% (w/v) sodium azide (Fisher Scientific Company, Fair Lawn, NJ). Cells were then stained by sequential incubations with: (1) saturating concentrations of mouse or rat mAbs; (2) biotin-conjugated $F(ab')_2$ affinity-purified goat anti-rat IgG (1:50) or biotin-conjugated $F(ab')_2$ affinity-purified rabbit anti-mouse IgG (1:100); and (3) Streptavidin-RED^{613} (1:50) on ice for 30 minutes. Cells were washed once between incubations and twice before analysis of fluorescence intensity using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

RESULTS

Effect of divalent cation on melanocyte attachment to ECM

Effects of single cations

In the absence of divalent cations, < 10% of melanocytes attached to ECMs (Fig. 1). However, 1 mM Mg^{2+}, near the physiological concentration (Mena, 1981; Menon et al., 1985), significantly enhanced melanocyte attachment to LM (44%), CIV (65.4%), FN (58.5%) and VN (80%); and 0.01 mM Mn^{2+}, near the physiological concentration in the skin (Mena, 1981), promoted melanocyte attachment to LM (40%), CIV (50%), FN (40%) and VN (70%). In contrast, Ca^{2+} had no effect on melanocyte attachment to LM and CIV at concentrations ≤ 100 mM, but it modestly promoted melanocyte attachment to FN at 100 mM (50%) and to VN at the more physiological concentration of 1 mM (65%).

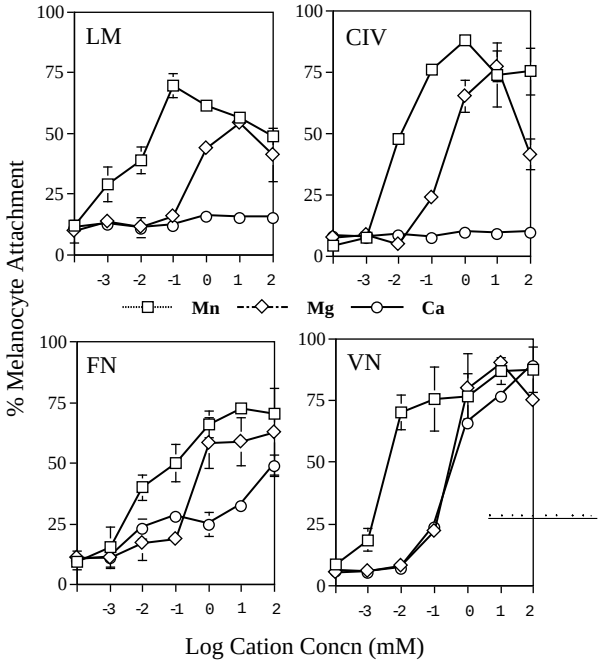

Fig. 1. Effect of divalent cations on melanocyte attachment to extracellular matrix molecules. ^{35}S-labelled melanocytes were incubated for 90 minutes in MAM supplemented with the indicated concentrations of $CaCl_2$, $MgCl_2$ or $MnCl_2$ in plates coated with 3 μg/cm^2 of LM, CIV, FN or VN. After removing the unattached cells by washing twice, attached cells were lysed and the radioactivity was counted with a beta counter. Percentage attachment was calculated by (recovered cpm of attached cells × 100)/(total cpm of plated cells). The results represent the mean ± s.d. of two experiments in triplicate are shown.

Effect of cation combinations

To determine possible synergistic effects of cations on melanocyte attachment to ECMs, melanocytes were plated on ECM in the presence of 1 mM $CaCl_2$, $MgCl_2$ and $MnCl_2$ alone or in combination (Fig. 2). Results for uncoated dishes and for the individual cations (1 mM) were virtually identical to those presented in Fig. 1 (data not shown). On all substrata melanocyte attachment in the presence of Mn^{2+} and Mg^{2+}, Mn^{2+} and Ca^{2+}, or all three cations, was comparable to that for Mn^{2+} alone. Melanocyte attachment to LM and CIV in the presence of Mg^{2+} plus Ca^{2+} were 35% and 25% lower, respectively, than for Mg^{2+} alone. However, their attachment to FN and VN was comparable to that for Mg^{2+} alone (Fig. 2).

Effect of temperature

Melanocyte attachment to ECMs in the presence of 1 mM $CaCl_2$, $MgCl_2$ or $MnCl_2$ was examined at 4°C and 37°C. On LM, CIV, FN and VN 42%, 54%, 84% and 90%, respectively, of melanocytes applied attached within 90 minutes at 37°C (data not shown). However, only 2-13% of melanocytes attached to these ECMs at 4°C. Melanocyte attachment to Poly-L, which is known to bind cells by its charge (Tawil et al., 1993), was unaffected by temperature (60% at 37°C versus 63% at 4°C).

Effect of ECM on dendrite outgrowth

Experiments to determine the effect of ECMs on melanocyte dendricity were performed in the presence of the lowest concentrations of the three cations (1 mM Ca^{2+}, 1 mM Mg^{2+} and 0.1 mM Mn^{2+}) previousely shown to mediate maximal melanocyte attachment. One, 2, 4, 8 and 24 hours after plating melanocytes on ECM-coated glass slides, photographs were taken of random fields. Cells possessing dendritic extensions were calculated as a percentage of the population.

Within four hours after plating, melanocytes had strikingly different morphologies on LM and CIV versus FN and VN (Fig. 3). On LM and CIV, >30% and >40% of the cells, respectively, were dendritic within 2-4 hours, while on FN and VN all cells assumed a bipolar or polygonal morphology (Figs 3, 4).

On LM approximately 50% of cells that were dendritic 4 hours after plating retracted their dendrites within 8 hours and 80% of the cells lost their dendrites within 24 hours (Fig. 4). In contrast, on CIV all initially dendritic melanocytes main-

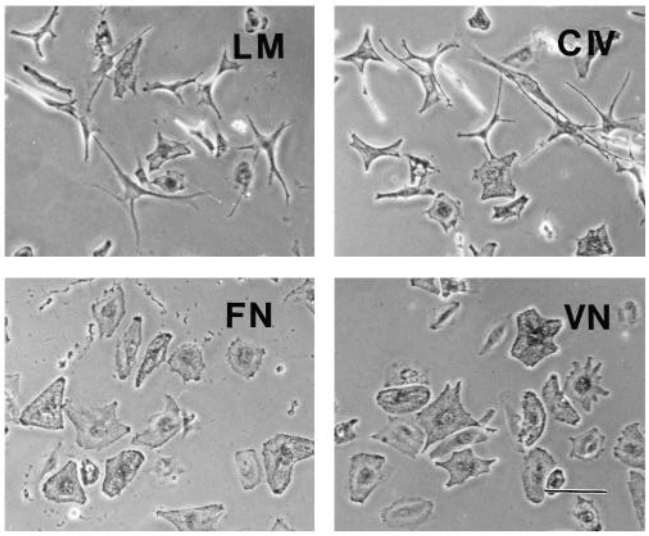

Fig. 3. Effect of substrata on melanocyte morphology. Phase-contrast micrographs of melanocytes 4 hours after plating on substratum-coated glass slides in the presence of 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1 mM $MnCl_2$. The majority of melanocytes display dendritic molphology on LM and CIV, with no striking difference between the length of dendrites on the two substrata, while those on FN and VN are polygonal. Bar, 50 μm.

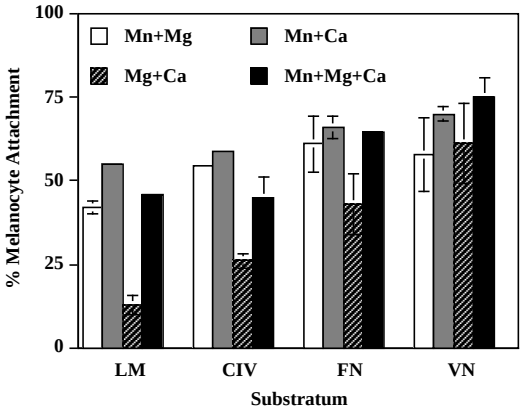

Fig. 2. Effect of divalent cation combinations on melanocyte attachment to extracellular matrix molecules. ^{35}S-labelled melanocytes were incubated on plates coated with 3 μg/cm^2 of LM, CIV, FN or VN for 90 minutes in combinations of 1 mM $CaCl_2$, 1 mM $MgCl_2$ or 1 mM $MnCl_2$. After removing the floating cells radioactivity from attached cells was counted. Percentage attached cells was calculated as described in Fig. 1. Mean ± s.d. from two experiments in triplicate is shown.

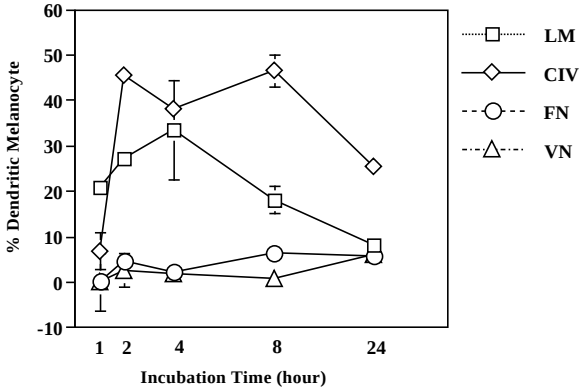

Fig. 4. Changes in melanocyte dendricity with time. Melanocytes were plated on different substratum-coated glass slides as before. The percentage of cells with one or more dendrites out of at least 300 melanocytes was calculated at 1, 2, 4, 8 and 24 hours after plating. Measurements were performed using ×100 magnification of phase-contrast micrographs. Dendrite outgrowth on LM and CIV occurred within 2 hours after plating. On LM 40% of dendrites retracted within 8 hours but on CIV most of dendrites were preserved for at least 8 hours, and approximately half of the dendrites were present even 24 hours after plating.

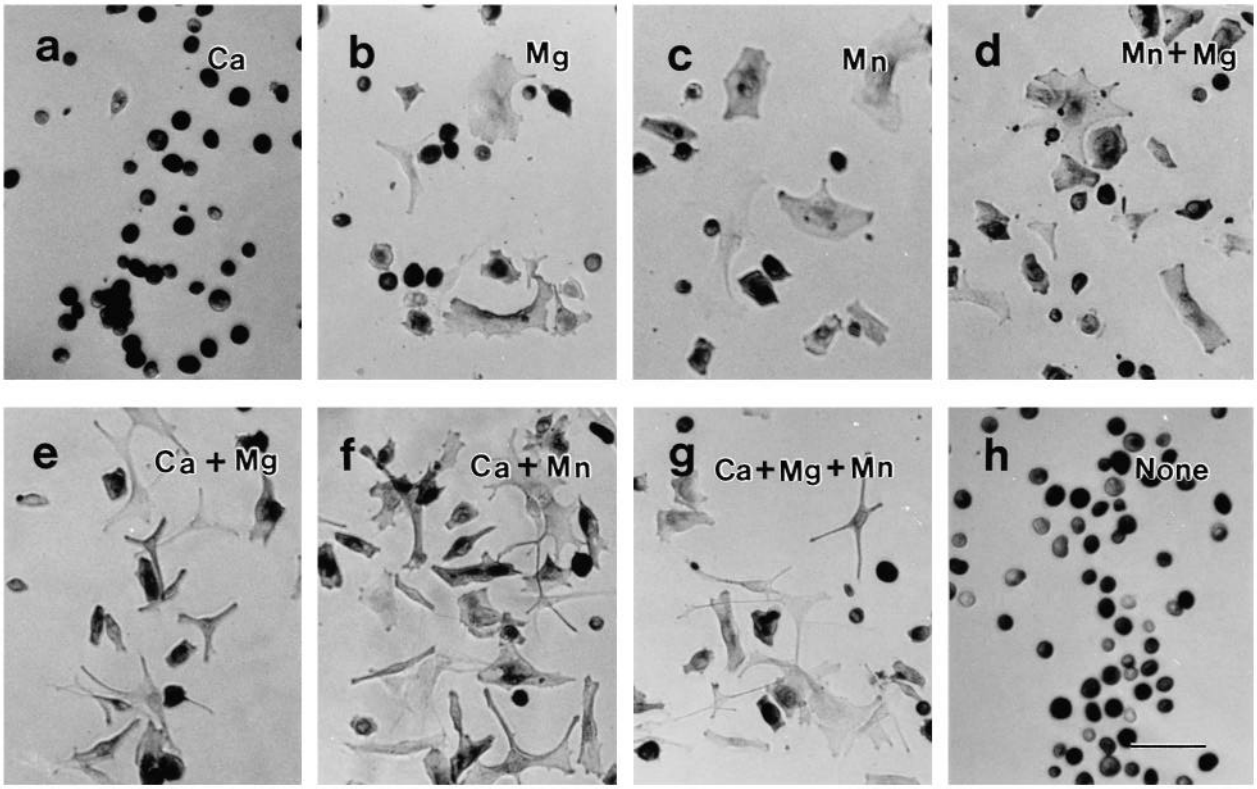

Fig. 5. Effect of divalent cations on melanocyte attachment and dendricity. Micrographs of melanocytes 4 hours after plating on LM-coated glass slides in the presence of one, two or three divalent cations. 1 mM Ca^{2+} alone (a) did not promote attachment or dendricity. 1 mM Mg^{2+} (b) or 0.1 mM Mn^{2+} (c) or both (d) promoted melanocyte attachment but did not promote dendrite formation. Combinations of Ca^{2+} and Mg^{2+} (e), Ca^{2+} and Mn^{2+} (f) or all three cations (g), resulted in formation of dendrites that were comparable in length in all three cation mixtures. In the absence of divalent cations (h) melanocytes did not attach. Bar, 50 μm.

tained their dendritic morphology for 8 hours and at least 50% of these cells remained dendritic even after 24 hours (Fig. 4).

Effect of divalent cations on melanocyte dendricity

To determine the role of cations in melanocyte attachment and dendrite formation, melanocytes were plated on LM or CIV in the presence of 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1 mM $MnCl_2$ alone or in combination. Cultures were photographed 4 hours after plating. Fig. 5 shows the representative melanocyte morphology on LM. Similar results were obtained with CIV (data not shown). The percentage of melanocytes with one or more dendrites, as defined in Materials and Methods, was calculated for attached melanocytes (Fig. 6).

Melanocytes plated on LM in the absence of divalent cations (Fig. 5h) or in the presence of Ca^{2+} alone did not attach (Fig. 5a), consistent with our previous attachment studies (Fig. 1). The round cells in Fig. 5 were floating and moved freely when the dishes were gently agitated. The majority of melanocytes plated in the presence of Mg^{2+} or Mn^{2+} or both attached to LM, but only 5-10% developed dendrites (Figs 5b-d,6). However,

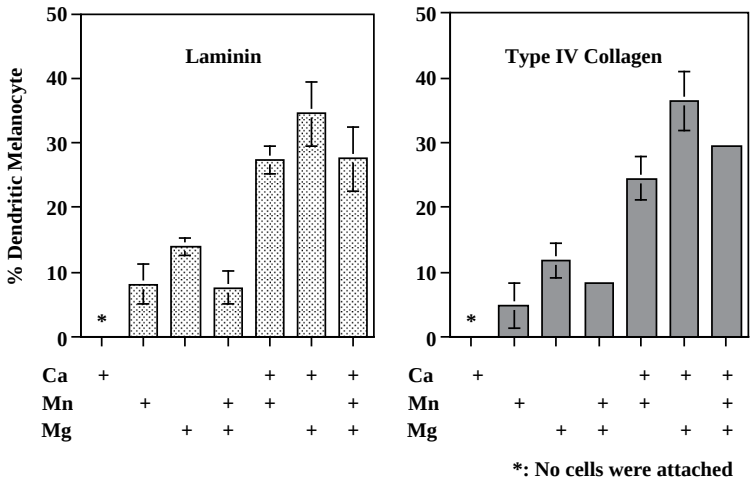

Fig. 6. Cation effects on melanocyte dendricity on LM and CIV. The effect of single or combinations of 1 mM Ca^{2+}, Mg^{2+} and 0.1 mM Mn^{2+} on melanocyte dendricity on CIV or LM were quantified. Percentages of dendritic melanocytes was calculated at 4 hours after plating on LM or CIV as described in Materials and Methods. The majority of melanocytes plated in the presence of Mg^{2+} or Mn^{2+} or both attached, but only 5-10% developed dendrites. However, 25-40% of melanocytes maintained in the presence of Ca^{2+} and either Mg^{2+}, Mn^{2+}, or both, became dendritic.

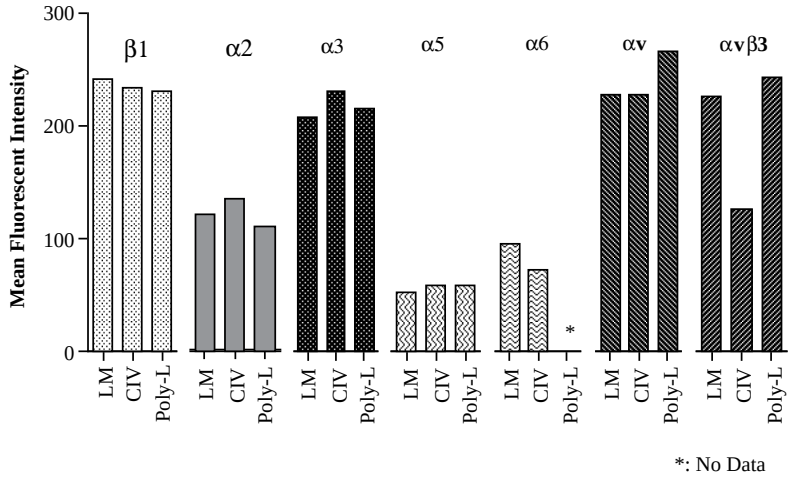

Fig. 7. Effect of substrata on integrin expression in melanocyte. Melanocytes on either LM, CIV or poly-L were collected 7 hours after plating by EDTA treatment and stained with different anti-integrin antibodies followed by biotin-conjugated second antibodies. Fluorescence intensities of cells were analyzed by flow cytometry. Results are displayed as mean fluorescence intensity. The expression of all integrins except for $\alpha_v\beta_3$ was comparable on the different substrata and on Poly-L.

25-40% of melanocytes developed dendrites in the presence of Ca^{2+} and Mg^{2+}, Mn^{2+} or both on LM (Figs 5e-g, 6).

Flow cytometry analyses of integrin expression

After 7 hours incubation on LM, CIV or Poly-L, melanocytes were detached by EDTA and were stained with saturating concentrations of mAbs against β_1, α_2, α_3, α_5, α_6, α_v or $\alpha_v\beta_3$ integrins. The cell suspensions were analyzed for fluorescent intensity using flow cytometry as described in Materials and Methods.

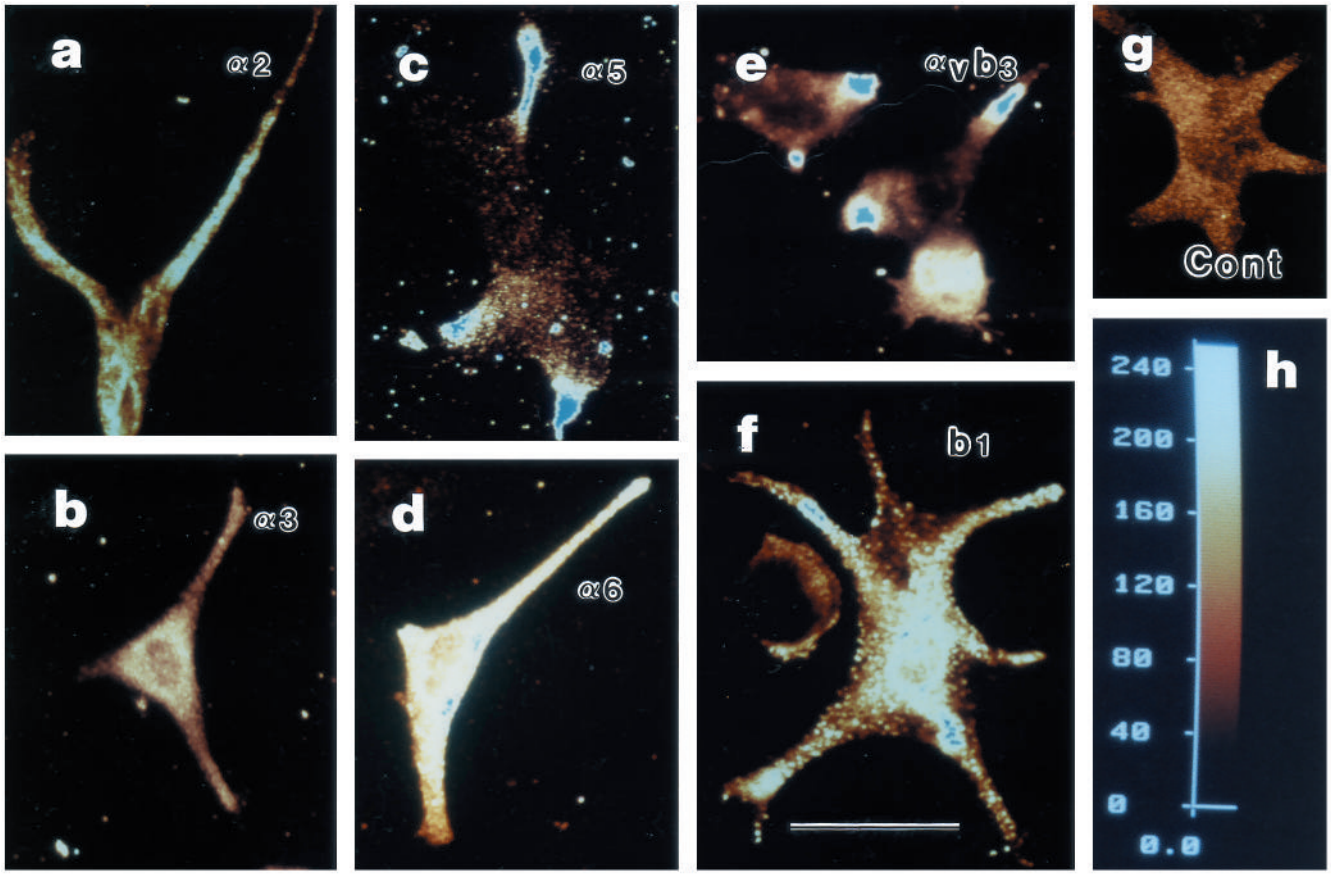

Fig. 8. Immunolocalization of integrins on melanocyte. Melanocytes were plated on glass slides coated with LM for 6 hours. Cells were then fixed in 4% paraformaldehyde/PBS and reacted with antibodies against α_2 (a), α_3 (b), α_5 (c), α_6 (d), $\alpha_v\beta_3$ (e) and β_1 (f) integrin subunits or heterodimers followed by FITC-conjugated second antibodies and analyzed by laser scanning confocal microscopy. The intensity of expression is expressed as a color gradient in which blue represents the highest and white the second highest intensity (h). The photographs display the cell-substratum interface. The α_2 subunit localized along the dendrites, while $\alpha_v\beta_3$ and α_5 localized along the middle and at the tip of the dendrites, respectively. The α_6 and β_1 were strongly expressed over the cell body and dendrites. The α_3 was only weakly positive. Control mouse IgG was negative (g). Bar, 50 μm.

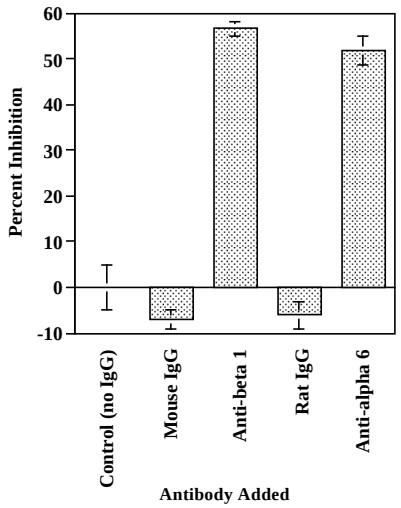

Fig. 9. Inhibition by anti-β_1 and anti-α_6 integrin antibodies of melanocyte attachment to laminin. Melanocytes were allowed to attach to microwells coated with LM in the presence of mAbs as well as control IgGs. After 90 minutes at 37°C, wells were rinsed to remove the nonadherent cells. The adherent cells were fixed and stained as described in Materials and Methods. Percentage attachment inhibition was calculated by (1–relative cell number in wells containing mAbs or irrelevant IgGs/relative cell number in wells without antibodies nor irrelevant IgGs)×100. The results represent the mean ± s.d. of two experiments in duplicate.

On all substrata melanocytes strongly expressed all integrins. The differences in mean fluorescence intensities among substrata for the different integrin subunits were minimal, except for the $\alpha_v\beta_3$ integrin heterodimer. $\alpha_v\beta_3$ expression on CIV was half that on LM or Poly-L (Fig. 7).

Immunolocalization of integrins on melanocytes

After 6 hours of incubation on LM- or CIV-coated 8-chamber glass slides, melanocytes were fixed with 4% paraformaldehyde and stained with mAbs against β_1, α_2, α_3, α_5, α_6 and $\alpha_v\beta_3$. The distribution of these integrins on dendritic melanocytes was analyzed by confocal laser scanning microscopy. Fig. 8 shows the distribution of fluorescent staining for each integrin in a narrow optical section through cells just above and parallel to the glass plating surface, thus corresponding to the cell-ECM interface. Similar results were obtained on both substrata, and photographs are shown for LM only.

α_6 and β_1 integrins were evenly distributed on the cell body and along the dendrites at the cell-LM interface (Fig. 8d,f). α_5 and $\alpha_v\beta_3$ integrins were strongly expressed at the tip of the dendrites (Fig. 8c,e) and α_2 was moderately expressed along the dendrites (Fig. 8a). α_3 Integrin was only weakly expressed at the cell-LM interface (Fig. 8b). Control mouse IgG was negative (Fig. 8g).

Effect of anti-integrin antibodies on melanocyte attachment and dendricity

To determine which integrins mediate melanocyte attachment and/or dendricity on LM and CIV, 5, 10 or 50 μg/ml of mAbs against β_1, α_2, α_3, α_5, α_6, α_v and $\alpha_v\beta_3$ integrins, all known to bind an integrin functional domain, were added individually to melanocytes at the time of plating. Dendricity was determined after 2 hours. LM and CIV showed similar results except for the attachment inhibition effect of mAbs against α_6 and β_1. These antibodies inhibited melanocyte attachment to LM by 52% and 57%, respectively, of a concentration of 50 μg/ml (Fig. 9), but did not block the attachment to CIV (data not shown). Pictures of melanocytes 2 hours after plating on LM are shown in Fig. 10.

Monoclonal antibodies against α_2, α_5, α_v and $\alpha_v\beta_3$ integrins did not prevent melanocyte attachment, but prevented dendrite outgrowth on both LM and CIV at concentrations of 5-50 μg/ml (Fig. 10a,c,e,f). Anti-α_3 antibody did not inhibit either dendrite formation or attachment on either substratum even at concentrations as high as 50 μg/ml (Fig. 10b). In the presence of 50 μg/ml of anti-α_6 or anti-β_1 mAb many of the melanocytes were still floating 2 hours after plating on LM (Fig. 10d,g), consistent with the result in Fig. 9. This attachment inhibition effect by anti-α_6 and anti-β_1 mAbs were not observed on CIV. Mouse or rat IgG, used as controls, had no effect on melanocyte attachment or dendrite formation even at the concentration of 50 μg/ml (Figs 9, 10h).

DISCUSSION

Integrin-mediated melanocyte interaction with ECM molecules, including LM, CIV, FN and VN, and the effect of divalent cations on these interactions, were investigated. Both LM and CIV promoted dendrite formation, while FN and VN did not. Mg^{2+} and Mn^{2+} were required for melanocyte attachment to all ECMs, but did not affect dendrite formation. In contrast, Ca^{2+} was required for dendrite formation on LM and CIV, but did not affect attachment to either substratum. We also found that attachment and dendrite formation on LM and CIV are processes mediated by different integrin heterodimers: $\alpha_6\beta_1$ affects melanocyte attachment to LM; and α_2, α_5 as well as $\alpha_v\beta_3$ integrins are involved in dendrite formation on LM and CIV. Interestingly, integrins that mediate dendrite formation tend to locate preferentially along or at the tip of dendrites, while integrins that mediate attachment tend to localize over the cell body as well as along the dendrites.

Our results are consistent with the known cation dependence of each integrin heterodimer. $\alpha_6\beta_1$ function is not dependent on Ca^{2+} (Hall et al., 1990; Sonnenberg et al., 1988, 1990), consistent with melanocyte attachment on LM independent of Ca^{2+}. Conversely, $\alpha_v\beta_3$ depends on Ca^{2+} to function (Leavesley et al., 1993), consistent with Ca^{2+}-dependent dendrite formation in melanocytes.

We have shown that both melanocyte attachment and dendrite formation require divalent cations. Ca^{2+} promoted melanocyte attachment to VN and FN (Fig. 1), suggesting that melanocytes express Ca^{2+}-dependent integrins that function in substratum attachment. It is not clear why Ca^{2+} did not affect melanocyte attachment to LM nor CIV (Fig. 1), since at least LM is known to contain domains that are recognized by almost all β_1 and β_3 integrin families including Ca^{2+}-dependent integrins (Kramer et al., 1990). One explanation is that Ca^{2+}-dependent integrins mediate only 'weak' attachment to LM and CIV, not strong enough to withstand the stringent washing procedure employed in our experiments. Easily disrupted bonds might be an advantage in mediating dendrite

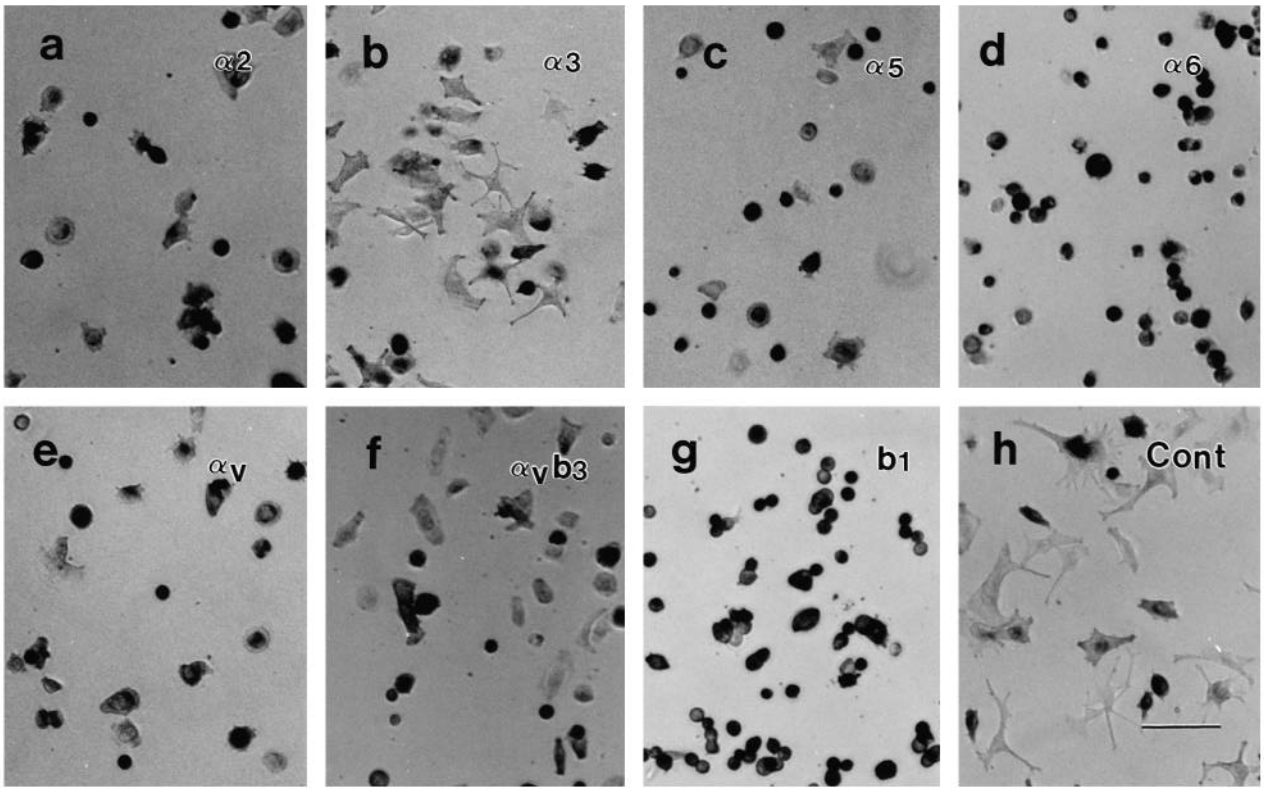

Fig. 10. Effect of anti-integrin antibodies on melanocyte attachment and dendricity. Melanocytes were plated on glass slides coated with LM in the presence of 50 μg/ml blocking anti-integrin antibodies. Representative fields are shown 2 hours after plating in the presence of anti-α_2 (a), anti-α_3 (b), anti-α_5 (c), anti-α_6 (d), anti-α_v (e), anti-$\alpha_v\beta_3$ (f) and anti-β_1(g) antibodies. Antibodies against α_2, α_5, α_v and $\alpha_v\beta_3$ did not block melanocyte attachment but blocked dendrite formation (a,c,e,f). Antibodies against α_6 and β_1 blocked many melanocyte attachments and also dendricity of attached cells (d,g). The round cells in (d) and (g) are floating. Antibody against α_3 (b) and control mouse IgG (h) did not inhibit attachment or dendrite formation. Bar, 50 μm.

extension, in that such cell-ECM connections must presumably be made and broken repeatedly as dendrites extend from the melanocyte cell body. Weak attachment is also consistent with the role of dendrites in transfering melanosomes from melanocytes to the large number of surrounding keratinocytes that compose the epidermal melanin unit (Hadley and Quevedo, 1966).

We found that the $\alpha_v\beta_3$ heterodimer localized to melanocyte dendrites (Fig. 8e). This heterodimer is unique among β_1 and β_3 integrin families in that it requires Ca^{2+} to function and cannot utilize other divalent cations (Leavesley et al., 1993). Interestingly, Clyman et al. (1992) showed that antibody against the $\alpha_v\beta_3$ integrin heterodimer did not block smooth muscle cell attachment to type I or IV collagen or to LM, although it blocked its migration on those substrata. As well, $\alpha_v\beta_3$ integrin affected cell attachment to VN, the recognized ligand for $\alpha_v\beta_3$. This observation seems to be comparable to our findings in that anti-$\alpha_v\beta_3$ integrin antibody inhibited dendrite formation of melanocytes on CIV and LM but did not block melanocyte attachment to those substrata (Fig. 10f). Furthermore, Ca^{2+} promoted melanocyte attachment to VN (Fig. 1), suggesting that the integrin activated by Ca^{2+} to promote attachment to VN may be $\alpha_v\beta_3$. On the basis of these data, we speculate that $\alpha_v\beta_3$ integrin can mediate strong binding such as required to sustain cell attachment to VN, but may be able to mediate 'weaker' attachment suitable for dynamic cell movement across ECM molecules like collagen or LM.

We deduce that $\alpha_2\beta_1$ and $\alpha_5\beta_1$ heterodimers also mediate melanocyte dendricity because antibodies directed against any one of these integrins (α_2, α_5 or β_1) blocked dendrite formation (Fig. 10a,c,g). Previously published data suggesting that $\alpha_5\beta_1$ binding occurs in the presence of any divalent cation and does not specifically require Ca^{2+} (Gailit and Ruoslahti, 1988; Kirchhofer et al., 1991), while $\alpha_2\beta_1$-mediated binding is inhibited by Ca^{2+} (Elices and Hemler, 1989; Staatz et al., 1989) may seem inconsistent with our finding that melanocyte dendricity was largely dependent on Ca^{2+}. We postulate, however, that the calcium dependence reflects the co-participation of $\alpha_v\beta_3$, known as noted above to require Ca^{2+} for its function. The apparent multiplicity of integrins involved in melanocyte dendricity is intriguing, given the hypothesis by Clyman et al. (1992) that β_1 integrins are primarily involved in anchoring cells while β_3 might instead be involved in cell movement, because β_1 integrin always co-localizes with vinculin, the linker protein to cytoskeleton, and β_3 does not. By analogy, $\alpha_2\beta_1$ and $\alpha_5\beta_1$ may play a role in stabilizing dendrites while $\alpha_v\beta_3$ may participate in their active extension.

We found several similarities in melanocyte attachment and dendrite formation on LM and CIV. On both substrata Mg^{2+} and/or Mn^{2+} were required for attachment, and Ca^{2+} was required for dendrite formation. Also, on both substrata α_2, α_5

and $\alpha_v\beta_3$ participated in dendrite formation; and Ca^{2+} appeared to inhibit Mg^{2+}-induced attachment (Fig. 2), possibly because substratum binding by $\alpha_6\beta_1$ is reported to be promoted by Mg^{2+} and inhibited by Ca^{2+} due to differences in molecular configulation produced by the cations (Sonnenberg et al., 1988, 1990). In contrast, anti-α_6 and -β_1 integrin antibodies blocked melanocyte attachment only to LM, suggesting that melanocyte attachment to LM but not to CIV is predominantly mediated by $\alpha_6\beta_1$ integrin. None of the anti-α_2, α_3, α_5, α_v or β_1 integrin antibodies as used singly inhibited melanocyte attachment to CIV. Possibly the attachment to CIV is mediated by other integrins and/or by combinations of many integrin heterodimers. As well, on CIV melanocytes retained their dendrites longer than on LM, suggesting that CIV may have more integrin recognition sites and/or more sequences for anchoring integrins and thus sustaining the dendrites.

FN and VN are ECM molecules reported to promote neurite outgrowth in some neural crest-derived cells (Rogers et al., 1983; Bozyczko and Horwitz, 1986; Neugebauer et al., 1991). In our experiments, neither FN nor VN promoted dendrite formation in melanocytes (Fig. 4). Possibly the effects of dendrite-mediating integrin(s) are masked by other integrins. In intact skin epidermal melanocytes are not in contact with either FN or VN, which are mainly present in the dermis; and dermally situated pigment cells (nevus cells) are not dendritic but rather polygonal in morphology, although in vitro they have the same substratum responsiveness as epidermal melanocytes (Yaar et al., 1988).

The specificity of melanocyte-ECM interactions was confirmed by temperature dependence, suggesting that integrins, similar to other adhesion molecules (Takeichi, 1977), require energy to aggregate at the plasma membrane where attachment occurs. Confocal laser scanning microscopy also suggests that this is the case, because integrins that mediated attachment or dendrite formation aggregated at the cell surface and were not evenly distributed over the entire cell-ECM interface. Moreover, integrins responsible for cell attachment (α_6 and β_1) tended to be distributed over both the cell body and the dendrites at cell-ECM interface, while those responsible for dendrite formation (α_2, α_5 and $\alpha_v\beta_3$) tended to localize to dendrites (Fig. 8).

We found that melanocytes attach to LM and CIV Ca^{2+}-independently and at least attachment to LM was mediated by the Ca^{2+}-independent integrin heterodimer ($\alpha_6\beta_1$), but dendrite formation requires Ca^{2+} and was mediated at least in part by Ca^{2+}-dependent integrins ($\alpha_v\beta_3$). It is interesting that in the epidermis there is a Ca^{2+} gradient, with a lower concentration of Ca^{2+} in the basal layer and progressively higher Ca^{2+} levels in the upper differentiated layers (Menon et al., 1985). It thus seems likely that in vivo melanocytes attach to the epidermal basement membrane through the Ca^{2+}-independent $\alpha_6\beta_1$ heterodimers and extend dendrites to the upper layers by interacting with an unrecognized ligand for the $\alpha_v\beta_3$ heterodimer as well as for α_2 and α_5 integrins. Overall, our data suggest a major role for specific integrin pairs in mediating melanocyte attachment and dendrite formation in skin.

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