Loss of adhesion to basement membrane components but not to keratinocytes in proliferating melanocytes

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We studied the adhesive characteristics of melanocytes, cultured either in the presence of the mitogen phorbol 12-myristate 13-acetate (PMA) that keeps them in a proliferative state, or in the absence of PMA allowing them to differentiate. On proliferating melanocytes, several integrins, ICAM-1, E-cadherin, and CD44 were expressed. In the absence of PMA, proliferation was arrested, melanin synthesis increased, and the morphology of the melanocytes became more spreaded. Under these conditions, expression of integrins αβ1 and αβ3 decreased, whereas expression of α2β1, α4β1, and α6β1 increased. No changes were observed for any of the other adhesion molecules. Immunoprecipitations from metabolically labeled cells confirmed the shift in integrin expression at the level of biosynthesis. The increased surface expression of α2β1 and α6β1 in the absence of PMA was accompanied by an induction of adhesion to basement membrane components collagen and laminin through these integrins. Integrin α5β1/αvβ3-mediated adhesion to fibronectin, CD44-mediated adhesion to hyaluronate, and E-cadherin/β1-integrin-mediated adhesion to keratinocytes were not affected by PMA. These findings indicate that by selective modulation of the expression of adhesion molecules, adhesion to components of the basement membrane is reduced in proliferating melanocytes, whereas adhesion to keratinocytes is maintained. Similar events may be involved in melanocyte proliferation and migration during wound healing and initial steps of melanocytic tumor progression.

Introduction

Melanocytes are neural crest-derived cells that are mainly situated in the basal layer of the epidermis where they make contact with the basement membrane and with adjacent keratinocytes. A single melanocyte distributes pigment to 25 to 35 keratinocytes through its dendrites, and such a cluster has been termed an “epidermal melanin unit” [13]. By production and transfer of melanin to keratinocytes, melanocytes protect keratinocytes from damage by UV radiation. In vitro, the morphology of melanocytes is controlled by contact with extracellular matrix (ECM) components [14], and keratinocytes control differentiation and proliferation of melanocytes [12, 40]. In vivo, normal melanocytes do not proliferate unless stimulated by environmental factors such as UV radiation or during processes such as wound healing [40]. Adhesive contacts with surrounding keratinocytes and the underlying basement membrane may help to conserve the non-proliferative state and characteristic morphology of melanocytes.

Molecules that mediate cell adhesion can be grouped in distinct families based on structural homologies between the members [18]. Integrins are αβ-heterodimeric cell surface glycoproteins that make heterophilic contacts with ECM proteins or with counterreceptors on other cells. Binding of integrins to their ligands can influence gene expression and control proliferation of cells [17]. Members of the immunoglobulin superfamily are cell surface glycoproteins made up of immunoglobulin repeats. They bind in a heterophilic manner to counterreceptors on other cells [4]. The E-selectin-ligand was recently found to be a variant of a fibroblast growth factor receptor [36]. Finally, a member of
the cartilage-link proteins, CD44, is a cell adhesion molecule involved in adhesion to hyaluronate (HA) and it is thought to act as a homing receptor on lymphocytes [23].

Integrin-mediated adhesion to ECM components and E-cadherin mediated binding to keratinocytes has been reported for melanocytes in vitro [38, 46]. In the present study, we investigated the adhesive characteristics of melanocytes cultured in the presence of the mitogen phorbol 12-myristate 13-acetate (PMA) that keeps them in a proliferative state, or in the absence of PMA allowing them to differentiate.

**Materials and methods**

**Materials and antibodies**

Laminin (Ln) and collagen type IV (ColIV), both isolated from Engelbreth-Holm-Swarm mouse sarcoma cells, were purchased from Life Technologies (Gaithersburg, MD/USA). Collagen type I (ColI) was isolated from rat tail. Fibronectin (Fn), isolated from human plasma, and hyaluronate (HA), isolated from human umbilical cord, were purchased from Sigma (St. Louis, MO/USA). The antibodies used are listed in Table 1.

**Isolation and culturing of melanocytes and keratinocytes**

Melanocytes were isolated from human foreskin epidermal cell suspensions by selective adherence and growth in Ham's F10 medium (Flow Laboratories, Irvine/UK) supplemented with 2% Ultroser-G synthetic serum (Life Technologies), glutamate, penicillin and streptomycin, 100 μg/ml penicillin and streptomycin (Sigma) and were cultured for a maximum of 3 passages in serum-free keratinocyte growth medium, and unattached cells were washed away. Subsequently, 32Cr-labeled melanocytes in Dulbecco's modified Eagle medium (DMEM) containing 0.25% BSA were seeded into the wells (10000 cells/well) and allowed to attach for 30 min at 37°C. After washing, PBS/BSA was added, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated F(ab')2 fragments of rabbit anti-mouse Ig antibodies (Dako, Glostrup/Denmark) or, in the case of GoH3 mAbs, with FITC-conjugated rabbit anti-rat Ig antibodies (Dako). Analyses were performed on an Epics Elite flow cytometer (Coulter, Mijdrecht/The Netherlands).

**Flow cytometry**

Cultured cells were harvested by short trypsinization of subconfluent monolayers and suspended in complete Ham's F10 medium. After washing with phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.02% azide, they were incubated with mAbs (Tab. I) in PBS/BSA/azide for 30 min at 4°C. After washing with PBS/BSA/azide, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated F(ab')2 fragments of rabbit anti-mouse Ig antibodies (Dako, Glostrup/Denmark) or, in the case of GoH3 mAbs, with FITC-conjugated rabbit anti-rat Ig antibodies (Dako). Analyses were performed on an Epics Elite flow cytometer (Coulter, Mijdrecht/The Netherlands).

**Immunoprecipitations**

Subconfluent monolayer cell cultures (75 cm²) were labeled overnight at 37°C with 0.5 mCi [³⁵S]methionine (Amersham, Houten/The Netherlands) in methionine-free medium (Flow Laboratories) containing 10% dialyzed fetal calf serum. The cells were washed 2 times with PBS and incubated with Nonidet P-40 (NP40) lysis buffer (0.5% NP40, 0.015 m NaCl, 0.01 m Tris, pH 7.5, 1.0 mM phenylmethylsulfonyl fluoride and 4 μg/ml aprotinin) at 4°C for 10 min. Subsequently the cells were scraped off the culture flask, repeatedly aspirated into syringes and forced through needles with decreasing diameter. Glycoproteins were isolated from NP40-solubilized cell extracts by adsorption to concanavalin A (Con A) Sepharose (Pharmacia Inc., Uppsala/Sweden). Immunoprecipitations were performed as described before [21], and samples were subjected to sodium dodecyl sulfate polyacrylamide gel eletrophoresis (SDS-PAGE) under reducing conditions. To compare the amount of glycoproteins in the various cell lines, equal numbers of cells of the Con A-bound fractions were used for immunoprecipitation.

**Cell adhesion assays**

Adhesion assays were performed as described before [8]. For adhesion to ECM components, polystyrene microtiter plates (96 flat-bottomed wells; Greiner, Alphen a/d Rijn/The Netherlands) were coated overnight with 20 μg/ml of the appropriate ECM component, washed and blocked with PBS/BSA. Coupling of HA to the wells was done as described before [42]. For adhesion assays to keratinocytes, these were allowed to adhere and spread in wells for 3 h in keratinocyte growth medium, and unattached cells were washed away. Subsequently, 32Cr-labeled melanocytes in Dulbecco's modified Eagle medium (DMEM) (Flow) containing 0.25% BSA were seeded into the wells (10 000 cells/well) and allowed to attach for 30 min at 37°C. After washing, the adherent cells were lysed in Triton X-100, and radioactivity was measure.
measured in a gamma-counter. For adhesion inhibition assays, melanocytes were incubated with mAbs 30 min at 4°C before being added to the wells, and keratinocytes were incubated with mAbs 30 min at 37°C prior to addition of labeled melanocytes.

**Results**

**Surface expression of adhesion molecules on cultured melanocytes**

Of the integrin family, proliferating melanocytes in the presence of PMA expressed predominantly integrins α3β1 and αvβ3 (Tab. II). Moderate levels of α5β1 and αvβ5 were expressed. Low expression was observed of α2β1, α6β1 and αvβ8, whereas α1β1, α4β1, α1β3 and β2- and β4-integrins were absent. Of the immunoglobulin superfamily only ICAM-1 was expressed, whereas ICAM-2, ICAM-3 and VCAM-1 were absent. No expression of E-selectin was detected, whereas high expression of E-cadherin was observed, and CD44 was very strongly expressed.

Removal of PMA from the medium induced a shift in morphology from a dendritic bipolar morphology to a more spreaded appearance (Fig. 1), it strongly increased melanin synthesis and it arrested proliferation (not shown). In the absence of PMA, surface expression of α3β1 and α5β1 decreased (Fig. 2). In contrast, faint expression of α4β1 became detectable and α6β1 expression was enhanced. Similarly, for melanocytes of 1 individual, enhanced expression of α2β1 was observed in the absence of PMA (Fig. 2), but this was not found for melanocytes from 2 other individuals (not shown). No differences in expression were observed between cells cultured with or without PMA for any of the other adhesion molecules (not shown).

These data show that arrest of proliferation and acquirement of a differentiated phenotype of melanocytes in the absence of PMA is accompanied by selective up- and down modulation of integrin expression levels.

**Synthesis of integrins in cultured melanocytes**

We next investigated if the shifts in the level of surface expression of α3β1 and α6β1 upon transition from a differentiated state to a proliferative state were reflected by changes in the level of biosynthesis of these integrins. Therefore, we immunoprecipitated the α3 and α6 chain from metabolically labeled melanocytes cultured in the presence or absence of PMA. In line with the surface expression data, synthesis of α3

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**Fig. 1.** Effect of PMA on the morphology of cultured melanocytes. Melanocytes were cultured in the presence (a) or absence (b) of PMA for two weeks.

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**Fig. 2.** Effect of PMA on the surface expression of adhesion molecules. Melanocytes were cultured in the presence (continuous line) or absence (dotted line) of PMA for two weeks and incubated with anti-integrin mAbs followed by FITC-labeled second antibodies. Fluorescence was measured on an Epics Elite flow cytometer. Results are shown for one out of three individuals.

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**Fig. 3.** Effect of PMA on the biosynthesis of integrins. Melanocytes of two patients were cultured in the presence or absence of PMA for two weeks as indicated. Subconfluent monolayers were metabolically labeled overnight, lysed, and glycoproteins were isolated by adsorption to Con A-sepharose. Equal amounts of counts from the Con A-bound fraction were used for immunoprecipitation with mAbs to α2, α3 or α6.
adhesion assays with melanocytes cultured in the presence or absence of PMA (Fig. 3). In melanocytes obtained from one individual we also observed induction of α2 synthesis in the absence of PMA but in melanocytes of another individual this was not the case (Fig. 3).

These results indicate that changes in surface expression of integrins upon removal of PMA from the culture medium are accompanied by changes in the level of biosynthesis of these molecules.

**Effect of PMA on adhesion of melanocytes to ECM components**

As removal of PMA from the culture medium affected the expression pattern of adhesion molecules, we performed adhesion assays with melanocytes cultured in the presence or absence of PMA. Melanocytes cultured with PMA adhered to Fn and HA but they adhered very poorly to Ln and not to Col or ColIV (Fig. 4a). However, after culturing in the absence of PMA for one week, melanocytes gained the ability to adhere strongly to Ln and Co, whereas adhesion to Fn and HA was unaffected. Adhesion to Fn was inhibited by mAbs to integrins α5β1 and αβ3 in the presence (Fig. 4b) or absence (not shown) of PMA. We have previously demonstrated that adhesion of melanocytes to HA is mediated by CD44 [42]. The enhanced adhesion to Ln of melanocytes grown in the absence of PMA could be blocked with anti-integrin β1 and anti-α6 mAbs but not with anti-α2 or anti-α3 mAbs (Fig. 4c). The enhanced adhesion to Ln of melanocytes grown in the absence of PMA could be blocked with anti-integrin β1 and anti-α6 mAbs but not with anti-α2 or anti-α3 mAbs (Fig. 4c). The induced adhesion to CoI and CoIV was blocked with anti-integrin β1 and anti-α2 mAbs and not with anti-α3 or anti-α6 mAbs. Interestingly, the induced α2β1-mediated adhesion to collagen was also observed for melanocyte cultures that showed no change in α2β1 expression.

These results indicate that, in line with loss of expression of α6β1 and α2β1, adhesion to Ln and Co through these integrins as observed for non-proliferative melanocytes, is absent from proliferating melanocytes in the presence of PMA.

**Effect of PMA on adhesion of melanocytes to keratinocytes**

As melanocytes expressed several receptors that have been implicated in cell-cell interactions in the epidermis (E-cadherin and integrins α5β1 and α3β1), we investigated adhesion to keratinocytes in the presence or absence of PMA. Melanocytes adhered to keratinocytes under both conditions (Fig. 5a). Removal of keratinocytes with EDTA and washing the wells 3 times with DMEM/BSA shortly before addition of the labeled melanocytes completely abrogated adhesion, indicating that ECM proteins produced by the keratinocytes and attached to the wells were not involved, and that indeed cell-cell interactions were studied. We used mAbs to integrin subunits α2 (5E8), α3 (P1B5) and β1 (4B4) and to E-cadherin (HECD-1) to inhibit adhesion. MAbs to integrin β1 and to E-cadherin moderately inhibited adhesion to keratinocytes and the combination of these mAbs inhibited adhesion for approximately 60% (Fig. 5b). No effect was observed of the mAbs to integrin α2 or α3 subunits.

These findings indicate that E-cadherin/β1-integrin-mediated contacts of melanocytes with proliferating, non-stratifying keratinocytes, are not influenced by PMA.

**Discussion**

In the present study, we have investigated the adhesive characteristics of cultured normal human melanocytes maintained either in a differentiated non-proliferative state (characterized by cell spreading, high level of melanin synthesis, and lack of cell division) or in a highly proliferative state induced by the phorbol ester PMA. Expression of adhesion molecules of the integrin-, immoglobulin-, cadherin-, selectin-, and CD44 families was determined, and the role of these receptors in adhesion to ECM components and to keratinocytes was explored.

The finding that melanocytes express predominantly α3β1 of the integrins and that they have E-cadherin expression is in line with earlier reports [38, 46]. We also detected expression of integrins α5β1 and αβ3 and of ICAM-1, three adhesion molecules that have been implicated in melanocytic tumor
progression [3, 7, 19, 26]. This is not due to any effect of the tumor promoter PMA since these molecules can also be detected in the absence of PMA. Expression may be induced by other culture conditions since these molecules are absent from melanocytes in vivo. The capacity of melanocytes to express such melanocytic tumor progression associated adhesion molecules may be important features during embryonic development and wound healing.

We have found that proliferating melanocytes adhere strongly to Fn but not or hardly to Ln and Co. This is probably due to the very low expression of α2β1 Ln/Co- and α6β1 Ln receptors in the presence of PMA and to the absence of αβ1 Ln/Co binding integrin. Even though α3β1 is a receptor for Ln, Co, and Fn, its high level of expression does not provide melanocytes with the capacity to adhere to Ln and Co, and α3β1 is not involved in the adhesion to Fn. It has been shown that α3β1 can be involved in migration of melanocytes [46]. In line with their findings, we have found that melanocyte adhesion to Fn is mediated by integrins α5β1 and αvβ3. However, others have shown that in neonatal but not fetal melanocytes, α3β1 can be involved in adhesion to Fn [32]. Such subtle discrepancies may possibly be explained by differences in culture conditions that may effect the activity of the adhesion molecules.

A dramatic effect of PMA is observed specifically on melanocyte adhesion to Ln and Co. In the absence of PMA, melanocytes adhere strongly to these basement membrane glycoproteins. Our finding that long-term culturing of melanocytes in the presence of PMA abrogates adhesion to Ln and Co is in line with findings for keratinocytes [1]. However, for keratinocytes this loss of adhesiveness is more general and opposite to its effect on melanocytes, PMA induces terminal differentiation of keratinocytes. Furthermore, in keratinocytes a general decrease of β1-integrin expression is observed, whereas melanocytes have a selective increase of α3β1 and α5β1 expression in the presence of PMA. Our immunoprecipitation data show that increased surface expression is accompanied by an increased level of synthesis. For other β1 integrins (α2β1, α4β1 and α6β1) we have found the opposite effect, namely that PMA selectively downmodulates surface expression and synthesis. In addition, even though modulation of α2β1 expression was only observed in melanocytes from one individual, in all cases we observed a strong effect of PMA on α2β1-mediated adhesion to Co. This suggests that PMA influences the activity of α2β1 as well. This effect is opposite to the effect observed when T-cells are incubated for a period of only several minutes with PMA [33]. Such a treatment enhances the affinity of β1 integrins on leukocytes, whereas long-term exposure of melanocytes to PMA appears to reduce the affinity of α2β1. Thus, PMA-induced proliferation of melanocytes selectively up- and downmodulates expression, synthesis and activity of integrins, which leads to a loss of adhesion to basement membrane components Ln and Co.

Our finding that melanocytes can use E-cadherin for binding to proliferating, non-stratifying keratinocytes, is in line with the report from Tang et al. [38], but we find that E-cadherin and β1-integrins are involved to approximately the same extent, whereas Tang et al. report a major role for E-cadherin. In our study and that of Tang et al., keratinocytes were maintained under the same conditions, but, again, different melanocyte culture conditions may influence the activity of the adhesion molecules involved. Even though α2β1 and α3β1 have been implicated in epidermal cell-cell interaction [22], up to 10 μg/ml of inhibitory mAbs to α2 (5E8), α3 (P1B5), β1 (4B4), E-cadherin (HECD-1) or combination as indicated. For (a) one experiment of three, and for (b) one experiment of two is shown. Means ± s.d. from triplicate wells are shown.

**Fig. 5.** Effect of PMA on adhesion to keratinocytes. - a. Melanocytes from two individuals were grown in the absence or presence of PMA for two weeks before being allowed to adhere to wells coated with increasing amounts of keratinocytes as indicated. In a parallel assay, keratinocytes were removed by EDTA and wells were washed prior to addition of the labeled melanocytes (dashed lines). - b. Melanocytes of two individuals were cultured in the presence of PMA and allowed to adhere to wells coated with 1.5 × 10^5 keratinocytes. Melanocytes and keratinocytes were incubated with 10 μg/ml of mAbs to α2 (5E8), α3 (P1B5), β1 (4B4), E-cadherin (HECD-1) or combination as indicated. For (a) one experiment of three, and for (b) one experiment of two is shown. Means ± s.d. from triplicate wells are shown.
might influence proliferation and migration of melanocytes during embryogenesis, wound healing, and the initial steps of melanocytic tumor progression.

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