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REGULATION OF INTEGRIN-MEDIATED ADHESION TO LAMININ AND COLLAGEN IN HUMAN MELANOCYTES AND IN NON-METASTATIC AND HIGHLY METASTATIC HUMAN MELANOMA CELLS

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We compared integrin-mediated adhesion to extracellular matrix (ECM) components of cultured human melanocytes and 6 human melanoma cell lines with different metastatic capacities in nude mice. Cultured melanocytes and most melanoma cell lines adhered strongly to fibronectin (FN), whereas only highly metastatic cell lines adhered to laminin (LM), collagen type I (COI) and type IV (COIV). Adhesion to LM and CO could be blocked by anti-α6 and anti-α2 monoclonal antibodies (MAbs) respectively. This observation is consistent with the finding that expression of LM receptor α6β1, and LM/CO receptor α2β1, was low on melanocytes and non- or poorly metastatic cell lines, whereas these integrins were strongly expressed on highly metastatic cell lines. In addition, immunoprecipitation from [35S]-methionine-labeled cells demonstrated increased synthesis of α6, α5 and β1 in highly metastatic cell lines and immunohistochemistry showed expression of α5β1 and α6β1 only in xenografts from highly metastatic cell lines. Furthermore, the observation that adhesion of melanocytes and non- or poorly metastatic cell lines could be stimulated with anti β1 MAbs demonstrates that these receptors, on these cells, are expressed in an inactive state. Our results suggest that α5β1 and α6β1 play a role in human melanoma metastasis in nude mice and demonstrate that interactions of these integrins with their ligands can be regulated at the level of surface expression and activation state of the receptor.

Crucial steps in the process of metastasis are the release and migration of cells from the primary tumor, penetration of the vessel wall, arrest in the microcirculation of distant organs and subsequent extravasation (Poste and Fidler, 1980). During this process the malignant cells interact several times with the ECM components of basal lamina and stroma including LM, CO and FN through specific receptors on the cell membrane. Interaction between cells and ECM components is mainly mediated by integrins.

Integrins consist of a superfamily of transmembrane receptors. Structurally, each integrin is a heterodimer consisting of an α subunit non-covalently bound to a β subunit. Based on the type of β subunit present in the dimer, the integrin superfamily can be subdivided into several families (Hynes, 1992). The most extensively studied are the β1, β2 and β3 families. Beta-1 integrins (very late antigens, VLA) play an important role in mediating cell-ECM contacts; β2 integrins are exclusively expressed on leukocytes and mediate cell-cell contacts whereas β3 integrins have a broader distribution and mediate cell-ECM interactions. In order to mediate their functions, integrins have to be in an activated state. For β1 integrins, this has been shown by Shimizu et al. (1990).

Integulin expression and adhesive behavior of cells can change after transformation. F2408 rat fibroblast and normal rat kidney (NRK) cells show reduced adhesion to FN after viral transformation (Plante Faber and Hynes, 1989) and certain human osteosarcoma cells show a changed pattern of integrin expression and of adhesive properties after further chemical transformation (Dedhar and Sautelner, 1990). Furthermore, transfection of the α2 gene has demonstrated the importance of VLA-2 in the metastatic process of rat rhabdomyosarcoma cells (Chan et al., 1991), while transfection of the α6 gene in M21 human melanoma cells that lack VNR expression has restored their tumorigenicity (Fielding-Habermann et al., 1992). Finally, VLA-2 (Bröcker et al., 1985; Klein et al., 1991), and the β3 subunit (Albelda et al., 1990), have been shown to be preferentially expressed in vertical-growth-phase primary melanoma lesions and melanoma metastases, suggesting a role in melanoma progression.

The purpose of this study is to investigate whether a correlation exists between the metastatic potential of human melanoma cell lines in nude mice and their capacity to adhere to ECM components in vivo, which integrins are involved and what the activation state of the integrins is on the various cell types.

MATERIAL AND METHODS

Cell lines and culture conditions

The melanoma cell lines used included: IF6 (Van Muijen et al., 1991a), 530 (Versteeg et al., 1988), M14 (Katano et al., 1984), Mel57 (Brüggen et al., 1978), BLM (Van Muijen et al., 1991a) and MV3 (Van Muijen et al., 1991b). All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Flow, Irvine, UK) supplemented with 10% FCS, gentamycin, glutamate and pyruvate. Isolation and propagation of human foreskin melanocytes were performed as described by Smit et al. (1989) and melanocytes were cultured for a maximum of 10 passages in Ham’s F10 (Flow) supplemented with 2% Ultra-ser-G synthetic serum (GIBCO, Grand Island NY) glutamate, penicillin, streptomycin, 0.1 mM IBMX (Sigma, St Louis MO) and 16 mM phorbol 12-myristate 13-acetate (PMA) (Sigma).

Monoclonal antibodies

The following anti-integrin subunit MAbs were used. TS2/7 anti-α1 (Hemler et al., 1984) (T-cell Sciences, Cambridge MA); A1.43 anti-α2 (Bröcker et al., 1985; Klein et al., 1991) (Cell Diagnostica, Münster, Germany); 5E8 anti-α2 (Zylstra et al., 1986); J143 anti-α3 (Fradet et al., 1984); GoH3 anti-α6 (Sonnenberg et al., 1987); 4B4 anti-β1 (Morimoto et al., 1985) (Coulter, Hialeah, FL); 8A2 anti-β1 (Kovach et al., 1992); TS2/16 anti-β1 (Hemler et al., 1984); 3E1 anti-β4 (Hesselt et al., 1984) (Telios, San Diego, CA). As negative controls in the FACS experiments, WT31 anti-CD3 (Spits et al., 1985; Tax et al., 1983) MAbs were used. As negative controls in the adhesion-inhibition assays, W6/32 anti-MHC class I (Barnst-
ble et al., 1978) MAbs were used. MHC class I is strongly expressed on all cell lines (Van Muijen et al., 1991a) and on cultured melanocytes (not shown).

**FACS analysis**

Cells were harvested by short trypsinization of subconfluent monolayers. After washing with DMEM containing 10% FCS, they were incubated with MAbs in PBS containing 0.5% BSA and 0.02% azide for 30 min at 0°C. After 3 washes 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 (Coulter, Mijdrecht, The Netherlands).

**Immunoprecipitations**

Subconfluent monolayer cell cultures (75 cm²) were labeled overnight at 37°C with 0.3 mCi [35S]-methionine (Amersham, Houten, The Netherlands) in methionine-free medium (Flow) containing 10% catalyzed FCS. The cells were washed twice with PBS and incubated with NP40 lysis buffer (0.5% NP40, 0.015 M NaCl, 0.01 M Tris pH 7.5, 1.0 mM phenyl-methyl-sulphonyl-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 of decreasing diameters. Glycoproteins were isolated from NP40 solubilized cell extracts by adsorption to concanavalin A (Con A) Sepharose (Pharmacia, Uppsala, Sweden). Immunoprecipitations were performed as described by Klein (1988). To compare the amount of glycoproteins in the various cell lines, equal numbers of counts of the Con-A-bound fractions were used for immunoprecipitation.

**Immunohistochemistry**

After acetone fixation, 4-μm frozen sections were incubated with MAbs for 1 hr at room temperature. After washing with PBS, bound MAbs were visualized using peroxidase-conjugated rabbit anti-(mouse Ig) or (in the case of GoH3 MAbs) with goat anti-(rat Ig) antibodies (Dako) and amino-ethyl carbazol (AEC). After counterstaining with Harris hematoxylin the sections were mounted with Kaizer's glycerin/gelatin.

**Cell adhesion assays**

LM and COIV, both isolated from Engelbreth-Holm-Swarm mouse sarcoma cells, were purchased from GIBCO. COI, isolated from rat tail, was a gift from Dr. E. Klein, Ulm, Germany. FN, isolated from human plasma, was purchased from Sigma. Polystyrene microtiter plates (96 flatbottomed wells; Greiner, Alphen a/d Rijn, The Netherlands) were coated by incubation overnight at 4°C with 50 μl of a solution containing 20 μg/ml of the appropriate adhesive protein in PBS. Unbound protein was removed by washing with PBS and free binding sites were blocked by incubation for 1 hr at 37°C with DMEM containing 0.25% BSA. Cells were harvested by short trypsinization of subconfluent monolayers, washed with DMEM containing 0.25% BSA and labeled in a volume of approx. 100 μl with 50 μCi Na[35S]CrO for 90 min at 37°C. Subsequently, the cells were washed 3 times, diluted to a concentration of 1 x 10⁵ cells/ml in DMEM containing 0.25% BSA and seeded into the wells (5,000 cells/well). Cells were allowed to attach for 30 min at 37°C in a 5% CO₂ atmosphere; non-adherent cells were removed by washing 3 times with DMEM containing 0.25% BSA; the attached cells were lysed with Triton-X-100 and radioactivity was measured in a gamma-counter. In adhesion inhibition/stimulation assays cells were incubated with the appropriate MAbs for 30 min at 4°C before seeding into the wells.

**RESULTS**

**Metastatic behavior of human melanoma cell lines in nude mice**

The rate of tumor take and the frequency of experimental and spontaneous metastasis of the human melanoma cell lines studied in nude mice has been described (Van Muijen et al., 1991a,b). Although, after s.c. inoculation, all cell lines gave a good tumor take, only BLM and MV3 were highly metastatic. IF6 and 530 did not metastasize at all and M14 and Mel57 were only poorly metastatic.

**Adhesion to ECM components**

To investigate whether the capacity to metastasize was reflected by the capacity to adhere to ECM components, we investigated adhesion to LM, COI, COIV and FN. Melanocytes, and the non-metastatic (IF6, 530) and poorly metastatic cell lines (M14, Mel57) adhered weakly to LM, COI and COIV whereas the highly metastatic BLM and MV3 melanoma cells adhered strongly to these ECM components (Fig. 1). A different pattern was found for adhesion to FN. Most cells exhibited strong binding to FN and only 530 cells adhered poorly to FN. These results indicate that highly metastatic melanoma cells adhere to LM and CO more strongly than non- or poorly metastatic cells.

**Expression of LM/CO receptors**

As adhesion to LM and CO was strongly increased in highly metastatic cell lines, surface expression of integrins, known to mediate adhesion to LM (α1β1, α2β1, α3β1, α6β1, α6β4) or CO (α1β1, α2β1, α3β1, α3β4) was investigated by means of FACS analysis. Melanocytes and all 6 cell lines expressed β1 integrins (Fig. 2). Expression of β1 was high on melanocytes and the highly metastatic BLM and MV3 cells, and low on the other cell lines. Expression of α2 was low, both on melanocytes and on all cell lines. Expression of α3 and α6 was high on the highly metastatic BLM and MV3 cells, whereas melanocytes and all other cell lines showed only weak expression or none at all. Expression of the α3 subunit was high on melanocytes and the highly metastatic BLM and MV3 cells, moderate on IF6 and Mel57 and low or undetectable on 530 and M14. No β1 expression was found on melanocytes or any of the cell lines, even though MAbs against β1 stained control cells (keratinocytes; not shown). From these results it can be concluded that the expression of α2β1 or α3β1 is strongly increased on highly metastatic melanoma cells compared to non- or poorly metastatic melanoma cells and normal melanocytes.

To establish whether differences in cell-surface expression were reflected by differences in the level of biosynthesis of integrin subunits, immunoprecipitation was performed on the glycoprotein fraction of [35S]-methionine-labeled melanoma cells. No α1, α2 or β1 could be precipitated from any of the cell lines and α3 synthesis was detected in all cell lines except 530 and M14 (Fig. 3). Consistent with the surface expression data, the level of synthesis of β1, α2, and α6 was increased in the highly metastatic BLM and MV3 cells as compared with the other cell lines.

Finally, we determined whether increased expression of LM/CO receptors also occurred in situ. Immunohistochemistry was performed on s.c. xenograft lesions from the non-metastatic cell lines IF6 and 530 and from the highly metastatic cell lines BLM and MV3. Expression of α2 and α6 was only detected in lesions of the highly metastatic BLM and MV3 cells (Fig. 4), as in the case of cultured cells.

**Role of α3β1 and α6β1 in adhesion to LM and CO**

To further demonstrate the role of α3β1 and α6β1 in adhesion to LM, COI and COIV we performed antibody-blocking studies. Since adhesion of normal melanocytes and the non-metastatic (IF6, 530) or poorly metastatic melanoma cells
Figure 1 – Adhesion of cultured human melanocytes (MCT) and six human melanoma cell lines to LM, COI, COIV and FN. Control adhesion to BSA was less than 5%. SD of triplicate determinations did not exceed 10%. One experiment out of 4 is shown.

(M14 and Mel57) to LM, COI or COIV was absent or low (Fig. 1), we only examined the highly metastatic BLM and MV3 cells. Adhesion of BLM and MV3 to LM could be inhibited by 60% by MAbs directed against α2 (Fig. 5), and MAbs directed against α6 or β1 completely blocked adhesion of these cells to LM. Adhesion to COI and COIV was almost completely inhibited by MAbs directed to α5 or β1. These results indicate that α5β1 and α6β1 are the major receptors for CO and LM, on highly metastatic melanoma cells.

Effect of stimulating anti β1 MAbs on adhesion to LM and CO

In order to determine whether differences in adhesion to LM and COI between cells of varying metastatic capacities were due not only to differences in expression of LM/CO receptors but also to the activation state of these receptors, we performed adhesion assays in the presence of 8A2 or TS2/16 anti-β1 MAbs. MAbs 8A2 and TS2/16 have been reported to stimulate integrin-mediated adhesion, possibly by a conformational change of the receptors. Following incubation with these MAbs, normal melanocytes and non- and poorly metastatic melanoma cells adhered strongly to LM and COI (Fig. 6). No stimulatory effects, or only minor ones, were observed with the highly metastatic BLM and MV3 cells (not shown). From these results we conclude that integrin receptors for LM and CO on normal melanocytes and non- and poorly metastatic melanoma cells are expressed in an inactive state, whereas these receptors are constitutively active in highly metastatic melanoma cells.

DISCUSSION

During metastasis, tumor cells interact at several sites with the ECM components of basal lamina and stroma. Therefore, we have compared the ability of cultured human melanocytes...
and human melanoma cell lines, having different metastatic capacities in nude mice, to adhere to LM, CO and FN.

No correlation between adhesion to FN and the metastatic capacity of the cell lines was found. Since most cells bound well to FN, a crucial role for FN in the metastatic process would not be expected. This observation confirms and extends the find-
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Figure 5 – Inhibition of adhesion of human melanoma cell lines BLM and MV3 to LM, COI or COIV. MAbs against α2 (5E8), α5 (GoH3), β1 (4B4) or combinations were added to the cells 30 min before being added to the wells. Data are expressed as percentage of remaining adhesion compared to parallel assays without MAbs. SD of triplicate determinations did not exceed 10%. One experiment out of 3 is shown. Incubation with W6/32 anti-MHC class-I control MAbs had no effect.

Figure 6 – Stimulation of adhesion of cultured human melanocytes (MCT) and 4 human melanoma cell lines to LM and COI with 8A2 anti-β1 MAbs. Control adhesion to BSA was less than 5%. SD of triplicate determinations did not exceed 10%. One experiment out of 3 is shown.

Findings of a number of previous studies in which no positive correlation was observed between the capacity of cells to adhere to FN and their tumorigenic and/or metastatic behavior (Dedhar and Saulnier, 1990; Terranova et al., 1984). Furthermore, cultured normal human melanocytes have been shown to adhere very weakly to LM and CO but strongly to FN (Gilchrest et al., 1985; this report).

An important role for LM in the metastatic behavior of melanoma cells has been reported (Terranova et al., 1982, 1984). Our data confirm these findings and show that (a) highly metastatic melanoma cells adhere strongly to LM; (b) this adhesion is mediated through VLA-6; and (c) expression of the LM receptor VLA-6 α-chain is markedly upregulated in highly metastatic human melanoma cells. Furthermore, our results indicate that adhesion of human melanoma cells to LM is mediated not exclusively through VLA-6 but also through VLA-2. Adhesion to COI and COIV has been demonstrated to be a property of various metastatic melanoma cell lines (Kramer and Marks, 1989). Our data confirm and extend these findings: the highly metastatic cells adhere strongly to COI and COIV whereas the non- or poorly metastatic cells and normal melanocytes adhere only weakly to these ECM components. Adhesion to both COI and COIV is mediated through VLA-2, which is strongly up-regulated in highly metastatic cells. These results are in agreement with the findings of Mortarini et al. (1991) who reported high α2 and α6 expression on tumor cells cultured from human melanoma metastases but not on tumor cells cultured from primary melanomas. Finally, a role for VLA-2 in melanoma tumor progression has been suggested by Bröcker et al. (1985) and Klein et al. (1991), who found that an increase in α2 expression in fresh human melanocytic lesions correlated with malignant progression. Our immunoprecipitation data indicate that elevated expression of α2 and α6 in highly metastatic cell lines is due not only to increased
It is well known that, in order to interact with their ligands, $\beta_1$ integrins have to be in an active state (Shimizu et al., 1990). MAbs have been described which induce a high-avidity state of VLA-2 and VLA-3, and which require interaction of a high-avidity state of $\beta_1$ integrins in order to adhere to LM and CO, whereas highly metastatic cells do not. Even though only a very low amount of VLA-2 and VLA-6 is expressed on non-metastatic cells, once these receptors are activated the cells adhere to LM or CO as strongly as do the highly metastatic cells. This suggests that the number of receptors is less important than the state of activity they are in.

In conclusion, we show that VLA-2 and VLA-6-mediated adhesion to LM and CO is strongly increased in highly metastatic human melanoma cell lines, and that this adhesion can be regulated at the level of (a) $\alpha_6\beta_{1}$ and $\alpha_6\beta_3$ expression, (b) $\alpha_6\beta_{1}$ adhesion and $\alpha_6\beta_3$ synthesis, and (c) activation of the $\alpha_6\beta_{1}$ and $\alpha_6\beta_3$ complexes.

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