Mitogenic Activity of Laminin on Human Melanoma and Melanocytes: Different Signal Requirements and Role of \(\beta 1\) Integrins

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ABSTRACT

The possible mitogenic activity of laminin (LN) on normal and neoplastic cells of the melanocytic lineage was tested by culturing growth-arrested human melanoma cells and neonatal foreskin melanocytes on LN. Serum-deprived, quiescent melanoma cells proliferated, in serum-free medium, in a dose-dependent fashion to immobilized LN as determined by \(^{3}H\)thymidine incorporation, cell cycle analysis, and change in cell number. The mitogenic activity of LN on melanoma cells was not mediated through autocrine release of growth factors and was observed with primary or metastatic melanoma cells and with clones isolated from the same metastasis but only on cells expressing very late antigen (VLA)-3 and VLA-6 laminin receptors. Proliferation of melanoma cells to LN was significantly inhibited by a mAb to the \(\beta 1\) subunit of VLA integrins and by a combination of mAbs to the \(\alpha\) subunits of VLA-3 and VLA-6. By contrast, LN did not act as a mitogen on human melanocytes expressing VLA-3 and VLA-6 and cultured in serum-free medium. However, a costimulatory activity of immobilized LN for proliferation of melanocytes was observed in the presence of a second signal provided by a set of different growth factors. The costimulatory activity of LN on melanocytes could be significantly inhibited by mAbs directed to the \(\alpha\) and \(\beta\) chain of VLA-6 but not to VLA-3. These data suggest that LN itself, and not growth factors possibly associated with it, can exert a mitogenic activity on quiescent human melanoma cells and that a change in the signal requirements for response to LN occurs upon neoplastic transformation in the melanocyte lineage. Furthermore, \(\beta 1\) integrins are differentially involved in the response of the normal and the neoplastic cells to LN, since VLA-3 and VLA-6 cooperate in the proliferation of neoplastic cells, while VLA-6 is relevant for the response of melanocytes.

INTRODUCTION

The interaction of normal and neoplastic cells with ECM components is thought to be a fundamental process in the regulation of cellular responses such as adhesion, spreading, migration, and gene expression (1–6). This interaction is largely, but not uniquely, dependent on the expression and function of integrins, a family of ECM surface receptors (1, 16, 17). Recent evidence also indicates that the integrin-dependent signals that can affect proliferation are transduced into the cell through the cytoplasmic domains of the \(\beta 1\) subunit (11).

One of the questions to be answered is whether different ECM components may provide signals influencing the proliferation of neoplastic cells (7–9). The available evidence supports the notion that, in normal cells, ECM components can promote cell proliferation by acting, not as direct mitogens, but as costimulatory signals (1, 10, 11). The costimulatory role of ECM components on normal cells is mediated through integrin receptors and is exemplified by the proliferative response seen in different subsets of T lymphocytes upon interaction with FN, LN, or vitronectin and simultaneous triggering of the T-cell receptor (12–14). In normal cells, the costimulatory activity of ECM components is thought to depend not only on ECM receptor expression but also on a high-affinity state of the integrin for the cognate ligand (1). The high affinity state of integrins for their ligands may be a constitutive condition that depends on the cell type expressing a given integrin (15). Alternatively, a high-affinity state of integrins is a transient phase induced by signaling through other cell surface receptors (1, 16, 17). Recent evidence also indicates that the integrin-dependent signals that can affect proliferation are transduced into the cell through the cytoplasmic domains of the \(\beta 1\) subunit (11).

Much less information is available on the possible mitogenic activity of ECM components on neoplastic cells (9, 18–20) and on the role of integrins in mediating this response. In a recent study, we provided evidence indicating that growth-arrested primary and metastatic melanomas can proliferate to FN and that the response was mediated through \(\alpha 5\beta 1\), a \(\beta 1\) integrin acting as a specific FN receptor (8).

However, it remains to be seen whether other ECM components, such as LN (21) and collagens, can exert an integrin-dependent mitogenic activity on human neoplastic cells. Furthermore, no direct comparison of normal and neoplastic cells from the same tissue has been attempted to see whether the process of neoplastic transformation can influence the possible mitogenic response to ECM components. To address these issues, normal human melanocytes and melanoma cells from primary and metastatic lesions were compared for possible proliferative response to LN, a major component of basement membranes. The results indicated that LN has a mitogenic activity on serum-deprived, quiescent human melanoma cells isolated from primary tumors and from metastatic lesions. The response was found to depend on the expression and function of two \(\beta 1\) integrin receptors, VLA-3 and VLA-6. By contrast, normal melanocytes, cultured in serum-free medium, did not proliferate to LN unless additional growth signals were provided. Furthermore, the costimulatory activity of LN on melanocyte proliferation was mediated mainly through the VLA-6 integrin receptor.

MATERIALS AND METHODS

Tumor Cells and Clones. Melanoma cell cultures were established at the Istituto Nazionale Tumori, Milan, from surgical specimens of primary lesions (Me5810, Me4405, and Me10538), lymph node (Me665/1, Me13443, and Me1811), or s.c. metastases (Me665/2 and Me9742/2) isolated from patients admitted for surgical treatment. Viable tumor cells were isolated from specimens as described previously (22). Melanoma cells were cultured in 75-cm\(^2\) tissue culture flasks (Costar, Cambridge, MA) in 10% heat-inactivated FCS (Pool Bioanalysis Italy, Milan, Italy) and RPMI 1640 (BioWhittaker, Walkersville, MD). Tumor clones were isolated from the s.c. metastasis Me665/2 at the fifth in vitro passage by a two-layer soft agarose technique (22). All melanomas used in this study grow as anchorage-dependent cells and do not proliferate in SFM. All melanoma cells and clones used in this study were checked by electron microscopy for the presence of pre-melanosomes and for the absence of Mycoplasma contamination. Mycoplasma contamination was also excluded by the Mycoplasma Detection Kit (Boehringer Mannheim Biochemica, Mannheim, Germany).
Human Melanocytes. Normal human melanocytes, isolated from neonatal foreskin, were cultured with a chemically defined medium as described (23). The melanocyte basal medium consisted of four parts of MCDB 153 (Sigma Chemical Co., St. Louis, MO) supplemented with 2 mm CaCl₂ and 1 part of Leibovitz’s L15 (Sigma). To obtain the melanocyte complete medium, the basal medium was then supplemented with 5 ng/ml EGF (Sigma), 40 μg/ml BPE (Sigma), 5 μg/ml insulin (Sigma), 10 ng/ml PMA (Sigma), and 2% FCS. The human foreskin melanocytes used in this study (FM937 and FM942) are pigmented cells, have a bipolar/spindle morphology, grow as anchorage-dependent cells, are not tumorigenic when injected into nude mice, do not grow in soft agar, have a normal diploid karyotype, and undergo senescence after a limited number of passages in vitro (24). Melanocytes also share a similar phenotype characterized by expression of markers normally lost upon neoplastic transformation as c-Kit (25) and ADAbp (26). The melanocytes also express low levels of the EGF and the nerve growth factor receptors (24) and other markers of the melanocyte lineage such as gp75 (tyrosinase-related), p97 (melanotransferrin), mCSP, and gp180 (24).

Indirect Immunofluorescence and mAbs. The expression of integrins on normal and neoplastic cells was analyzed by flow cytometry as described (22) using a FACSscan and the Lysis II software (Becton Dickinson, Mountain View, CA). The following mAb to different integrin subunits were used: 4B4 (IgG1), anti-β1 (Coulter, Hialeah, FL); TS2/7 (IgG1), anti-α1 (T Cell Diagnostics, Cambridge, MA); Gi9 (IgG1) (Immunotech, Marseille, France) or P1E6 (IgG1) (Oncogene Science, Manhasett, NY), anti-α2; P1B5 (IgG1), anti-α3 (Oncogene Science); 2H6 (IgG1), anti-α5 (10); GoH3 (IgG2a), anti-α6 (Immunotech); AMF7 (IgG1), anti-αv (Immunotech); and ZZ21 (IgG1), anti-β3 (Immunotech). For some experiments, an anti-ICAM-1 mAb (clone 84H10, IgG1, Immunotech) was also used.

Proliferation Assay. Tumor cells were kept for 24–48 h in medium without FCS (SFM) to block their proliferation and then were seeded at 1–2 × 10⁵ cells/well in 0.2 ml SFM in flat-bottomed 96-well plates (Costar 3595) coated previously with purified mouse LN (#L2020, Sigma, or #23017–015 Gibco, Life Technologies, Gaithersburg, MD) or human LN (#12163–010, Life Technologies). Purity of all LN preparations, as indicated by the suppliers, was >95%. Coating was accomplished as follows. The plates were incubated overnight at 4°C with various concentrations of LN (ranging from 100 to 0.09 μg/ml) in 0.2 ml PBS. Unbound controls were incubated with PBS alone. Excess unbound LN was washed out, and the plates were incubated for 1–2 h at 37°C, 5% CO₂. Nonspecific binding to BSA-coated wells ranged from 6 to 11% for all tumors. Results of adhesion assays with ⁵¹Cr-labeled cells were expressed as % adhesion (cpm from bound cells/cpm from total input of cells × 100).

RESULTS

Quiescent Human Melanoma Clones in SFM Proliferate to LN. As reported recently (8), the growth of melanoma clones isolated from the s.c. metastasis Me665/2 can be blocked by culture in RPMI 1640 without FCS (SFM). The efficacy of this SFM in inducing a quiescent state in melanoma cells was confirmed by additional experiments with uncloned primary and metastatic melanomas (8). We have also shown that tumor cells cultured in SFM drastically reduce their thymidine incorporation and withdraw from the S and G₂-M phases of the cell cycle after only 24 h without FCS (8).

To test the possible mitogenic activity of immobilized LN on human melanoma, tumor clones isolated from Me665/2 were “made” quiescent by a 24-h culture in SFM and then seeded on LN-coated wells. Fig. 1 shows that two of the four clones (2/14 and 2/60) could proliferate in a dose- and time-dependent fashion to immobilized LN.
while clone 2/60 reached the highest proliferation at 48 h (Fig. 1). The optimal response for both clones was observed at the highest dose both immobilized and soluble LN (Fig. 2), although immobilized LN clones; clone 2/14 had a peak response at 72 h after seeding on LN, of LN, but a slightly different kinetics was evident between the two clones. The response to LN was evaluated after 48 h by pulsing the cells with 1 pCi [3H]thymidine/well during the last 8 h of culture. Results are expressed as stimulation index. Proliferation of melanoma clones 2/14 and 2/60 to LN shown in A and B is significantly different from control proliferation without LN at the LN concentrations of 100, 25, 6.2, and 1.6 µg/ml (clone 2/14) or at the LN concentrations of 100, 25, and 6.2 µg/ml (clone 2/60; SNK test, P = 0.01). The horizontal line across the histograms represents the control proliferation observed in the absence of LN. Bars, SD.

The optimal response for both clones was observed at the highest dose of LN, but a slightly different kinetics was evident between the two clones; clone 2/14 had a peak response at 72 h after seeding on LN, while clone 2/60 reached the highest proliferation at 48 h (Fig. 1). Proliferation of melanoma clones to LN was observed in response to both immobilized and soluble LN (Fig. 2), although immobilized LN induced a higher response than soluble LN. The proliferative response of melanoma cells to LN was obtained both with mouse and human LN from three different sources (data not shown).

The culture of melanoma cells on immobilized LN also induced progression through the cell cycle. As shown in Table 1, in comparison to cells growing exponentially in medium supplemented with 10% FCS, the proportion of cells in the S phase of the cell cycle was reduced from 37.7 to 7.4% after a 24-h culture in SFM and declined further to 3.2% after 48 h in SFM. By contrast, in cells kept in SFM for 24 h and then cultured on LN-coated flasks, the proportion of cells in S phase increased to 17.5% after 8 h and reached a peak value of 39.5% after 32 h. By 48 h on LN-coated plates, 11.7% of the cells were in the G2-M phase, and this proportion became 15.1% at 72 h. The kinetics of cell cycle progression from G1 to S to G2-M phases, observed by culturing on LN, was slower than that observed in 10% FCS but similar to that induced by culturing on FN (Table 1; Ref. 8). The same tumor cells were also checked for the change in cell number. As described recently (8), culture in SFM induces a steady reduction in cell number with almost complete cell death ensuing after 144 h. In contrast, cells kept on LN-coated wells underwent a doubling between 96 and 144 h after initial seeding on this ECM component (data not shown). The integrin profile (Table 2) indicated that melanoma clones 2/14, 2/60, 2/21, and 2/43 expressed different subunits of LN receptors. Although most integrin subunits of LN receptors (β1, α1, α2, α3, αv, and β3; Refs. 28 and 29) were found on all clones, expression of α6, the α-chain of a specific LN receptor (α6β1/VLA-6), was observed only on the two clones (2/14 and 2/60) that proliferated to LN, thus suggesting a possible involvement of VLA-6 in the response.

Taken together, these data indicate that LN can exert a direct mitogenic activity on quiescent melanoma cells and that the response is subjected to clonal variation within the same neoplastic lesion.

Mitogenic Activity of LN on Human Primary and Metastatic Melanomas: Correlation with Integrin Profile. The mitogenic activity of LN and the correlation with expression of VLA-6, observed with melanoma clones, were evaluated with a panel of uncloned primary and metastatic melanomas isolated from different patients. When cultured on LN-coated plates, quiescent cells from primary melanomas (Me5810 and Me4405) and from metastatic melanomas (Me13443 and Me665/1) proliferated in a dose-dependent fashion to immobilized LN. Tumor clones cultured for 24 h in SFM, then were seeded (1 X 10⁶/culture) on wells coated with 100 (), 25 (0), 6.2 (u), 1.6 (n), 0.39 (c), and 0.09 (@) µg/ml LN from three different sources (data not shown). The integrin profile (Table 2) indicated that melanoma clones 2/14, 2/60, 2/21, and 2/43 expressed different subunits of LN receptors. Although most integrin subunits of LN receptors (β1, α1, α2, α3, αv, and β3; Refs. 28 and 29) were found on all clones, expression of α6, the α-chain of a specific LN receptor (α6β1/VLA-6), was observed only on the two clones (2/14 and 2/60) that proliferated to LN, thus suggesting a possible involvement of VLA-6 in the response.

Table 1 Cell cycle analysis of melanoma clone 2/60 cultured on LN or FN in SFM

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>G1</th>
<th>S</th>
<th>G2-M</th>
</tr>
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<tbody>
<tr>
<td>10% FCS (48 h)</td>
<td>37.4</td>
<td>37.7</td>
<td>24.9</td>
</tr>
<tr>
<td>SFM (24 h)</td>
<td>88.5</td>
<td>7.4</td>
<td>4.1</td>
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<td>SFM (48 h)</td>
<td>94.9</td>
<td>3.2</td>
<td>1.9</td>
</tr>
<tr>
<td>SFM (72 h)</td>
<td>90.5</td>
<td>6.9</td>
<td>2.6</td>
</tr>
<tr>
<td>SFM (24 h) → LN (8 h)</td>
<td>80.1</td>
<td>17.5</td>
<td>2.4</td>
</tr>
<tr>
<td>SFM (24 h) → LN (24 h)</td>
<td>70.9</td>
<td>28.1</td>
<td>1.0</td>
</tr>
<tr>
<td>SFM (24 h) → LN (32 h)</td>
<td>59.3</td>
<td>39.5</td>
<td>1.2</td>
</tr>
<tr>
<td>SFM (24 h) → LN (48 h)</td>
<td>63.6</td>
<td>24.7</td>
<td>11.7</td>
</tr>
<tr>
<td>SFM (24 h) → LN (72 h)</td>
<td>64.0</td>
<td>20.9</td>
<td>15.1</td>
</tr>
<tr>
<td>SFM (24 h) → FN (24 h)</td>
<td>81.7</td>
<td>17.3</td>
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</tr>
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<td>66.6</td>
<td>21.7</td>
<td>11.7</td>
</tr>
<tr>
<td>SFM (24 h) → 10% FCS (24 h)</td>
<td>35.7</td>
<td>35.5</td>
<td>28.8</td>
</tr>
<tr>
<td>SFM (24 h) → 10% FCS (48 h)</td>
<td>51.0</td>
<td>30.7</td>
<td>18.3</td>
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</table>

The percentage of cells in G1, S, G2-M phases of the cell cycle was determined by cytofluorimetric analysis as described in “Materials and Methods” by acquisition of 20,000 events.

*Exponential growth in 10% FCS-RPMI 1640 for 48 h.
† Growth in SFM for 24 h, 48 h, 72 h.
‡ Growth in SFM for 24 h and then on LN (plates were pre-coated with 100 µg/ml of LN) for 24 h* 48 h, 32 h†, 72 h‡.
§ Growth in SFM for 24 h and then on FN (plates were precoated with 100 µg/ml of FN) for 24 h or 48 h.
¶ Growth in SFM for 24 h and then in 10% FCS-RPMI 1640 for 24 h or 48 h.

Table 1 Cell cycle analysis of melanoma clone 2/60 cultured on LN or FN in SFM

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‡ Growth in SFM for 24 h and then on LN (plates were pre-coated with 100 µg/ml of LN) for 24 h, 48 h, 32 h, 72 h.
§ Growth in SFM for 24 h and then on FN (plates were precoated with 100 µg/ml of FN) for 24 h or 48 h.
¶ Growth in SFM for 24 h and then in 10% FCS-RPMI 1640 for 24 h or 48 h.
Proliferation of Melanoma Cells to Laminin

Fig. 6 shows that the mitogenic activity of LN on human melanomas can be observed with primary and metastatic tumors, is subjected to intertumor heterogeneity, correlates with the expression of VLA-6, and is not dependent only on the process of adhesion.

VLA-3 and VLA-6 Cooperate in the Proliferative Response of Melanoma Cells to LN. To obtain information on the role of integrins in mediating the proliferative response of melanoma cells to LN, inhibition experiments were carried out with mAbs directed to distinct \( \alpha \) and \( \beta \) integrin chains. Preliminary experiments (Fig. 5) allowed us to identify the optimal mAb concentration (1 \( \mu \)g/ml) that, in a 48-h adhesion assay, did not inhibit cell spreading and adhesion to LN. Then, using the 1 \( \mu \)g/ml mAb concentration, two melanoma clones (2/14 and 2/60) and one uncloned tumor (Me5810) were tested for proliferation to LN after preincubation with a mAb to the \( \beta 1 \) chain or, as negative control, with a mAb to \( \alpha 5 \), the \( \alpha \) subunit of VLA-5, a receptor involved in the recognition of fibronectin and not of LN. Data in Fig. 6 indicate that the mitogenic activity of LN on human melanomas could be completely abrogated by treating tumor cells with the anti-\( \beta 1 \) mAb but not by preincubation with the anti-\( \alpha 5 \) mAb. The finding that the anti-\( \beta 1 \) mAb can inhibit melanoma proliferation to LN, without inhibiting long-term adhesion to the same ECM component, supports a signaling role of \( \beta 1 \) integrins in mediating the mitogenic activity of LN on melanoma cells.

The role of the \( \alpha \) subunits of different VLA integrins was then studied using the 1 \( \mu \)g/ml anti-VLA-6 mAb concentration. Preliminary experiments (Fig. 5) suggested that the mAb was effective only when used at this concentration. The role of \( \alpha \) subunits was assessed in the presence or absence of LN at 50 \( \mu \)g/ml (data not shown). As shown in Table 2, all tumors expressed \( \alpha 1 \), \( \alpha 2 \), \( \alpha 3 \), \( \alpha 6 \), and \( \beta 3 \). However, the \( \alpha 6 \) subunit was expressed only on the four tumors that proliferated to LN (Me5810, Me4405, Me13443, and Me665/1) but was absent from the three tumors (Me1811, Me9742/2, and Me10538) that did not respond to LN.

Table 2: Expression of integrin subunits on melanoma clones from Me665/2

<table>
<thead>
<tr>
<th>Tumor clone</th>
<th>( \beta 1 )</th>
<th>( \alpha 1 )</th>
<th>( \alpha 2 )</th>
<th>( \alpha 3 )</th>
<th>( \alpha 6 )</th>
<th>( \alpha v )</th>
<th>( \beta 3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2/14</td>
<td>93( ^a )</td>
<td>85</td>
<td>68</td>
<td>91</td>
<td>60</td>
<td>97</td>
<td>34</td>
</tr>
<tr>
<td>2/60</td>
<td>94</td>
<td>74</td>
<td>50</td>
<td>96</td>
<td>90</td>
<td>97</td>
<td>76</td>
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<tr>
<td>2/21</td>
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<td>2/43</td>
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<td>46</td>
<td>20</td>
<td>86</td>
<td>6</td>
<td>97</td>
<td>70</td>
</tr>
</tbody>
</table>

\( ^a \) Results of cytofluorimetric analysis with mAbs to different integrin subunits are expressed as percentage of positive cells.

LN, while cells from two additional metastases (Me1811 and Me9742/2) and from one primary tumor (Me10538) did not respond (Fig. 3). As seen with melanoma clones, even primary and metastatic melanomas expressed different integrin subunits of LN receptors (see the table below the graph in Fig. 3). In fact, all tumors expressed \( \beta 1 \), \( \alpha 1 \), \( \alpha 2 \), \( \alpha 3 \), \( \alpha v \), and \( \beta 3 \). However, the \( \alpha 6 \) subunit was expressed only on the four tumors that proliferated to LN (Me5810, Me4405, Me13443, and Me665/1) but was absent from the three tumors (Me1811, Me9742/2, and Me10538) that did not respond to LN.

The lack of proliferation to LN by tumors Me1811, Me9742/2, and Me10538 was confirmed by further proliferation assays performed at 72 and 120 h (data not shown) and was not due to lack of adhesion to LN. In fact, as shown in Fig. 4, all three nonproliferating melanomas were able to adhere to LN in a dose-dependent fashion, and one of them (Me1811) bound to LN as efficiently as the four proliferating tumors (Me665/1, Me4405, Me5810, and Me13443). In addition, proliferative response was LN specific since culture of the same seven melanomas on a different ECM protein (type IV collagen) did not result in any stimulation of growth (data not shown). These data indicate that the mitogenic activity of LN on human melanomas can be observed with primary and metastatic tumors, is subjected to intertumor heterogeneity, correlates with the expression of VLA-6, and is not dependent only on the process of adhesion.
clone 2/60 (30), did not affect the response to LN. Similar results were
observed in the absence of mAbs pretreatment (SNK test, P = 0.01).

investigated by pretreating tumor cells with mAbs directed to α1, α2,
α3, and α6 chains. As shown in Fig. 7, while the anti-β1 mAb again
abrogated the response to LN, none of the anti-α chain mAbs was
effective when used alone. However, almost complete inhibition of
proliferation to LN was obtained by a simultaneous pretreatment of
tumor cells with two different mAbs directed to the α3 and α6
subunits, and no further reduction of proliferation was observed by
combining three different mAbs (anti-α2, anti-α3, and anti-α6; Fig.
7). No inhibition of proliferation was observed by any other combi-
nation of anti-α chain mAb, and no visible effect of inhibition of cell
spreading was observed by either anti-β1 or anti-α3 plus anti-α6
mAbs (data not shown). Furthermore, preincubation of tumor cells
with an anti-ICAM-1 mAb, an antigen highly expressed on melanoma
clone 2/60 (30), did not affect the response to LN. Similar results were
obtained by inhibiting the proliferation of uncloned primary and
metastatic melanoma cells as well as using mouse or human laminin
from three different sources (data not shown). Additional experiments
also indicated that the inhibition of proliferation to LN, obtained by
the combined use of anti-α3 and anti-α6 mAbs, was not due to
inhibition of cell adhesion to LN. As shown in Fig. 8, inhibition of
proliferation of melanoma clone 2/60 to LN was obtained either by
preincubating the tumor cells with the anti-α3 plus anti-α6 mAbs
before seeding the cells on LN or by adding the same mAbs to cells
already adherent and spread on LN.
Proliferation of Melanoma and Melanocytes to Laminin

Fig. 9. Proliferative response of melanoma cells to LN is not mediated through release of soluble growth factors. Melanoma cells (clone 2/60, clone 2/21, Me9742/2, and Me10538) seeded on wells coated (+) or not (−) with LN (100 μg/ml) were cultured in SFM for 48 h in the presence of a 1:2 dilution of supernatants produced previously by culturing clone 2/60 on LN-coated wells (B and D) for 8 (●), 24 (●), and 48 h (●). ●, the proliferative response of the different tumor cells to LN in the absence of supernatants. Tumor cells were also cultured in the presence of supernatants (A and C) from clone 2/60 cultured for 8 (●), 24 (●), and 48 h (●) without LN coating. Proliferative response of tumor cells to LN and/or to supernatants was evaluated after 48 h by pulsing the cells with 1 μCi [3H]thymidine/well during the last 8 h of culture. The horizontal line across the histograms represents the control proliferation observed in the absence of LN coating and in the absence of supernatants. The proliferative responses of all tumor cells to clone 2/60 supernatants are not significantly different from response in the absence of supernatants. Proliferative response of clone 2/60 to LN is significantly different from response in the absence of LN coating (SNK test, \( P = 0.01 \)).

Taken together, these data indicate that the mitogenic signal of LN on melanoma cells is mediated by \( \beta 1 \) integrins and that two receptors, VLA-3 and VLA-6, cooperate in the response.

The Mitogenic Activity of LN on Melanoma Cells Is Not Mediated Through Release of Growth Factors. The mitogenic signal delivered through \( \beta 1 \) integrins might induce melanoma proliferation through induction of growth factors. To test this hypothesis, the supernatants of a LN-responsive tumor (clone 2/60) produced by culturing tumor cells for 8, 24, or 48 h on wells coated or not with LN were tested for direct mitogenic activity on four different melanomas including clone 2/60 and three LN-nonresponsive tumors (clone 2/21, Me9742/2, and Me10538). As shown in Fig. 9, no significant induction of proliferation by any of the supernatants was seen on either the VLA-3\(^{-}/\)VLA-6\(^{-}\) clone 2/60 or on three different VLA-3\(^{+}/\)VLA-6\(^{-}\) melanomas normally unable to respond to LN. Furthermore, the supernatants from LN-cultured clone 2/60 failed to exert any mitogenic effect, even when added to cells seeded on LN (Fig. 9). Taken together, these data indicate that growth factors are not involved in the proliferative response of melanoma cells to LN and are in agreement with a direct role of \( \beta 1 \) integrins as the necessary and sufficient requirement for response of neoplastic cells to LN.

LN Acts as a Costimulatory Signal for Proliferation of Human Melanocytes. The proliferative response to LN, observed with primary and metastatic melanomas in SFM, might be dependent only on the expression of the appropriate LN receptors or might also be related to the transformed phenotype of these cells. To address this issue, we verified whether normal human melanocytes, cultured in SFM, may or may not proliferate to immobilized LN. Human melanocytes isolated from neonatal foreskin have a limited life span in vitro and can proliferate only in the presence of a set of growth factors (23–24). Previous results indicated that removal of all these factors from the culture medium results in a 90% reduction in \(^{[3H]}\) thymidine incorporation after only 24 h and in extensive cell death ensuing after 196 h (24). Two different early melanocyte cultures were then deprived of all the exogenous factors and cultured in SFM to test for proliferation to immobilized LN. Results shown in Fig. 10 indicate that two different melanocytes (FM937 and FM942) when cultured in SFM could not proliferate to LN. In addition, no proliferation to LN could be observed, even when the SFM was supplemented with only one of the four main exogenous factors (EGF, PMA, BPE, or insulin; Fig. 10). However, when the same four factors were combined together (EPBI medium), a marked mitogenic response to LN could be ob-
pretreating melanocytes with an anti-α6 mAb. Furthermore, no inhibitory effect was evident when using an anti-α3 mAb that, instead, produced a slight but significant increment of proliferation. Again, in contrast to the results seen with melanomas, no further inhibition was obtained, in comparison to the effect of anti-α6 alone, by combining the anti-α3 and the anti-α6 mAbs. Finally, a mAb directed to the β3 integrin chain did not affect the proliferation of melanocytes to LN (Fig. 11). Additional inhibition experiments, performed by combining anti-α1, anti-α2, anti-α3, and anti-α6 mAbs did not reveal any additional blocking effect in comparison to the inhibition seen by blocking the VLA-6 receptor (data not shown). Taken together, these results indicate that β1 integrins are involved in the proliferative response of melanocytes to LN. However, while the response of melanoma to LN depends on a cooperation between VLA-3 and VLA-6, in melanocytes the costimulatory activity of LN appears to be mediated mainly through VLA-6.

**DISCUSSION**

The results of this study indicate that LN can exert a mitogenic activity on quiescent melanoma cells and on normal melanocytes and that β1 integrins mediate the proliferative response to this ECM protein. However, normal and neoplastic cells showed profound differences in the signal requirements for the response to LN. Normal melanocytes did not proliferate to LN in SFM, unless a set of additional exogenous growth signals was provided. Thus, in melanocytes, as in other normal cells, LN acts as a costimulatory signal for proliferation. By contrast, melanoma cells did not require additional exogenous signals for proliferation to LN. Furthermore, in melanoma cells two different integrins, VLA-3 and VLA-6, cooperated in the proliferative response to this ECM.

The results of this study should be interpreted in the light of the most recent evidence on the signaling function of integrins. The available evidence indicates that signaling through integrins involves two distinct processes known as “inside-out signaling” and “outside-in signaling” (1, 15, 17, 31–35). Recent evidence indicates that the outside-in signaling function of integrins can play a role in cellular proliferation and that this process is strictly dependent on expression and function of the integrin β1 chain (11). In agreement with the proposed role of the β1 chain in signaling for proliferation, the

![Fig. 10. LN acts as a costimulatory signal for proliferation of melanocytes. Melanocytes were cultured for 24 h in SFM (without EGF, PMA, BPE, insulin, and FCS) and then seeded (1 × 10^6) on wells coated with LN. The SFM was supplemented or not with only one of four factors (EGF, PMA, BPE, or insulin) or with all of them (EPBI). The response to LN was evaluated after 48 h by pulsing the cells with [3H]thymidine/well during the last 8 h of culture. Proliferative response of melanocytes to LN in EPBI medium is significantly different from response in the same medium without LN (SNK test, P = 0.01). Bars, SD.](image1)

<table>
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<th>Table 3 Expression of integrin subunits on melanocytes</th>
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<td><strong>Integrin profile</strong></td>
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* Results of cytofluorimetric analysis with mAbs to different integrin subunits are expressed as percentage of positive cells.

![Fig. 11. Inhibition of costimulatory activity of LN on melanocytes by mAbs to integrin subunits. Melanocytes were cultured for 24 h in SFM (without EGF, PMA, BPE, insulin, or FCS) and then were preincubated or not with mAbs 4B4 (anti-β1), P1BS (anti-α3), GoH3 (anti-α6), or with P1BS plus GoH3 (anti-α3 + anti-α6), and finally the cells were seeded (3 × 10^4) on wells coated with LN in the presence of SFM or of EPBI medium (SFM supplemented with EGF, PMA, BPE, and INS). Proliferative response of melanocytes in the presence of anti-β1 or anti-α6 mAbs or in the presence of anti-α3 + anti-α6 mAbs is significantly different from control proliferation to LN (EPBI medium) in the absence of mAbs (SNK test, P = 0.01). Proliferation of melanocytes to the complete medium in the absence of LN (1300 cpm ± 157) became 1278 cpm ± 192 and 1206 cpm ± 124 in the presence of anti-β1 or anti-α6, respectively.](image2)

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response of melanoma cells to both FN (8) and LN (this study) was found to be mediated through β1 integrins.

As for melanoma proliferation to FN (8), we obtained a similar inhibition of response by exposing tumor cells to anti-integrin mAbs either before or after adhesion and spreading on LN. In addition, blocking of melanoma cell proliferation to LN by anti-integrin β1 chain mAb was obtained in conditions that did not interfere with long-term adhesion to LN. These results suggest that the mAb-mediated inhibition of response to LN is not simply mediated through inhibition of adhesion. In fact, interference with function of integrin receptors led to the inhibition of proliferation, even when adhesion and spreading had already occurred or in the absence of a detectable inhibition of adhesion. Not only that but proliferation to LN was obtained in response to either immobilized or soluble LN, although the response to immobilized LN was more efficient. Therefore, although the process of adhesion to LN may be an important step in the tumor-ECM interaction, nevertheless the proliferation does not appear to occur only as result of cell adhesion. This interpretation is strongly supported by the observation that three melanomas that did not proliferate to LN, nevertheless were able to adhere to this ECM protein.

In melanoma clones and in uncloned tumors, we found a correlation between proliferation and expression of VLA-6, but the inhibition experiments indicated that the mitogenic activity of LN could be blocked only by interfering at the same time with both VLA-3 and VLA-6 receptors. These data indicate that in melanoma, both receptors cooperate in the interaction with LN and that the signal for proliferation to LN has a threshold that requires the engagement of both β1 integrins. This interpretation may explain why the VLA-3 receptor, expressed also on tumors that do not respond to LN, may not be sufficient for inducing the proliferative response when VLA-6 is not expressed.

It is known that the neoplastic transformation in the melanocyte lineage is associated with the de novo expression of a LN receptor, known as α7β1, not found on melanocytes and playing a role in the adhesion of melanoma to this ECM protein (36). We have not tested the tumors used in this study for expression and function of α7β1 and, therefore, we cannot exclude a role of this receptor in mediating the proliferative response of melanoma cells to LN. However, the results of blocking experiments indicated that the response could be abolished by anti-VLA-3 and anti-VLA-6 mAbs, suggesting that these two receptors are the main mediators of the response. Furthermore, it is to be considered that interaction of different cell types with LN may be mediated not only by integrins but also by other families of receptors (reviewed in Ref. 37), including a high-affinity protein known as 67 Kd receptor (38). However, phenotype analysis of tumor cells used in this study by the 67 Kd LN receptor-specific mAb MluC5 (39) indicated only a very weak expression on some tumor lines with no correlation with response to LN (data not shown).

Data on the integrin profile of melanocytes indicate that both VLA-3 and VLA-6 are expressed in vivo (40, 41). However, the VLA-3 receptor did not appear to play a role in the response of normal melanocytes to LN. In fact, inhibition of proliferation was obtained only by interfering with VLA-6, and no additional effect was found by combining anti-α3 and anti-α6 mAbs. The differential involvement of VLA-3 in response to LN, between melanoma and melanocytes, suggests that neoplastic transformation is associated with a change in the role of this receptor. The mechanism that mediates this process is unknown, but it may be related to the outside-in signal transducing ability of integrins, a function that is dependent on the activation state of the receptor. It is conceivable that factors controlling the constitutive affinity state of integrins may be differentially expressed in normal and neoplastic cells. The identification of these factors will allow us to verify whether they are causally involved in the constitutive regulation of the affinity state and in the outside-in signaling function of integrins expressed in normal and neoplastic cells of the melanocyte lineage.

The contrast between the mitogenic activity of LN on SFM-cultured melanomas and the costimulatory role of the same ECM protein on melanocytes represents a clear-cut distinction between the biological responses of normal and neoplastic cells to the same extracellular signal. In the normal cells, the mitogenic activity of LN was observed only when a set of at least three exogenous factors (as combination of insulin, EGF, and BPE or as combination of EGF, PMA, and BPE) was added to the SFM. In these cells, it cannot be excluded that the integrin-mediated signal (dependent mostly on VLA-6) might result not only from VLA-6 expression but also from integrin activation. Integrin activation is known to be induced by PMA (42–43) that is used for in vitro growth of melanocytes. However, as shown by the experiments with SFM supplemented with single exogenous factors, PMA alone was not able to induce the proliferative response to LN. Furthermore, proliferation of melanocytes to LN could be observed by combining EGF, insulin, and BPE, without PMA. Therefore, a multisinusial model, with LN playing a costimulatory role mediated through VLA-6, may explain the response of melanocytes to this ECM component.

The commercially available LN preparations may contain trace amounts of growth factors. These contaminants may contribute to the observed mitogenic activity of LN. However, the lack of mitogenic activity of LN on melanocytes, even when in the presence of one of four different exogenous factors (EGF, PMA, BPE, or insulin) strongly argues against a role of contaminants in LN preparations as responsible for the mitogenic activity of this ECM protein.

Melanoma cells proliferated to LN in the absence of any exogenous factor, thus suggesting that signaling through two integrin receptors (VLA-3 and VLA-6) is sufficient to trigger the response in the neoplastic cells. This possibility is in agreement with the observation that integrin ligands can stimulate the kinase activities associated with p34/cdc2 and cyclin A (20). In addition, c-fos and c-jun, two genes thought to be involved in the regulation of cell proliferation, can be rapidly and transiently induced by LN peptides able to stimulate growth of PC12 cells (9). These data indicate the existence of direct signaling pathways linking integrin receptors to intracellular growth regulators and support the hypothesis that LN acts as a mitogen for VLA-3/VLA-6+ melanomas.

An alternative possibility to be investigated is that LN acts as a costimulatory signal for proliferation in both normal and neoplastic cells. "Second signals" might be provided in vivo to the neoplastic cells by growth factors associated with ECM (44), or released by normal cells surrounding the neoplastic population (45), or even produced by tumor cells (45–48). Constitutive expression of multiple growth factors in melanoma but not in melanocytes has been described (49). In addition, in a recent report, RNA transcripts for IL-1α, IL-1β, IL-5, IL-6, leukemia-inhibitory factor, IL-7, IL-8, Rantes, IL-10, IFN-β, tumor necrosis factor-α, granulocyte-colony-stimulating factor, granulocyte/macrophage-colony-stimulating factor, EGF, endothelial cell growth factor, basic fibroblast growth factor, and nerve growth factor were found in most or all melanomas, including the cell lines used in this study, but not in melanocytes (50). However, the experiments with supernatants from LN-stimulated melanoma cells provided evidence against a role of soluble growth factors in the response LN. In fact, these supernatants did not exert any mitogenic activity, not only on tumor cells normally unable to respond to LN, but even on the tumor clone 2/60 used to produce them. Furthermore, no induction or increase of proliferation was observed, even when adding the supernatants
to melanoma cells seeded on LN, indicating the absence of any cooperative or synergistic effect of LN and soluble factors released by an autocrine mechanism in response to this ECM. Therefore, it appears that in melanoma the expression of the relevant receptors (VLA-3 and VLA-6) is the only requisite for proliferation LN.

Finally, the evidence on mitogenic activity of LN on human melanoma, together with the previous data on the similar role of FN (8), indicate that interaction of tumor cells with different ECM components is a significant factor in promoting the growth of neoplastic populations expressing the relevant ECM receptors. The identification of β1integrins as key receptors in this process suggests that effective block of the growth-promoting activity of ECM components on human melanoma can be attempted by interfering with integrin functions.

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Mitogenic Activity of Laminin on Human Melanoma and Melanocytes: Different Signal Requirements and Role of $\beta_1$ Integrons

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