Expression of activated leukocyte cell adhesion molecule (ALCAM)/CD166 correlates with the aggregation and metastatic capacity of human melanoma cell lines (Am J Pathol 1998, 152:805–813). Immunohistochemistry on a series of human melanocytic lesions reveals that ALCAM expression correlates with melanoma progression. Most nevi (34/38) and all thin melanomas studied (Clark levels I and II) did not express ALCAM. In contrast, immunoreactivity was detected in the invasive, vertical growth phase of 2 of the 13 Clark level III lesions tested. The fraction of positive lesions further increased in Clark level IV (13/19) and in Clark level V (4/4) lesions. ALCAM expression was exclusively detectable in the vertical growth phase of the primary tumor. In melanoma metastases, approximately half of the lesions tested (13/28) were ALCAM positive. According to the Breslow-thickness, ALCAM expression was observed in less than 10% of the lesions that were thinner than 1.5 mm and in over 70% of the lesions that were thicker than 1.5 mm. Our results strongly suggest that ALCAM plays an important role in melanocytic tumor progression and depict it as a new molecular marker for neoplastic progression of primary human melanoma. (Am J Pathol 2000, 156:769–774)
lines, and ALCAM expression correlated with the ability to form clusters of cells. Here we extend the association of ALCAM expression with advanced neoplastic progression stages of human melanoma cell lines to human primary MM. Immunohistochemistry on a large series of fresh human melanocytic lesions revealed an increased expression with MM progression. We propose that ALCAM represents a new molecular marker for the progression of primary human melanoma, with a possible prognostic value.

**Materials and Methods**

**Tissue Specimens**

The 121 biopsies used in this study were collected after surgical removal of pigmented skin lesions for diagnostic purposes. They comprised 38 benign lesions (ie, 30 nevocellular nevi, 3 Spitz nevi, 2 ordinary nevi, and 1 cellular blue nevus and 2 cases of benign lentigo), 55 MMs, and 28 MM metastases. The primary MMs included 38 superficial spreading MMs (8 Clark level I, 9 Clark level II, 11 Clark level III, 10 Clark level IV), 4 lentigo MMs (2 Clark level I, 2 Clark level III), 6 acro lentiginous MMs (3 Clark level IV, 3 Clark level V), and 7 nodular MMs (6 Clark level IV, 1 Clark level V). The MM metastases were excised from the skin (11 cases) or lymph node (17 cases). All biopsies were received fresh, and representative parts were frozen in liquid nitrogen-cooled isopentane.

**Immunohistochemistry**

Five-μm frozen sections were air-dried, fixed for 10 minutes in absolute acetone, and stained with a three-step avidin-biotin complex method, using anti-ALCAM/CD166 antibody (clone 18; Antigenix America Inc., NY) as primary reagent at a final concentration of 10 μg/ml. The biotinylated rabbit-anti-mouse Ig and avidin-biotin complex were purchased from Dako (Glostrup, Denmark). All incubations were carried out at room temperature for 30 minutes and followed by three washes in phosphate-buffered saline. The reaction product was visualized with aminoethylcarbazole as a substrate and hydrogen peroxide; the brightly red-stained immunoreactive sites could easily be distinguished from melanin pigment. The sections were briefly counterstained with Harris’ hematoxylin and mounted with glycerin jelly.

**Statistical Analysis**

The Fisher’s exact test for small sample numbers was used for statistical analysis.

**Results**

In normal human skin, ALCAM expression was observed in the inner hair sheath and in the bulb of anagen hair follicles, the arrector pili muscle, as well as the clear cells of eccrine sweat glands (Figure 1a) and, weakly, the acrosyringium. Variable staining was observed in macrophages. Small nerve fibers in the papillary dermis were immunoreactive, whereas larger nerves in the deep dermis did not stain. Langerhans’ cells showed variable immunoreactivity. Keratinocytes and endothelial cells of dermal blood vessels were negative. In contrast to a previous report, perivascular fibroblasts did not display immunoreactivity. The immunohistochemistry data are summarized in Table 1. The majority of benign lesions (34 of the 38) did not show any reactivity in the nevus cells (Figure 1b). A weak and granular cytoplasmic positivity was observed in one congenital nevocellular nevus and in the junctional component of one acral compound nevus. The dermal component of these compound lesions was negative. A dermal nevocellular nevus with neviod differentiation showed weak cytoplasmic positivity in the areas of neurotoid metaplasia (Figure 1c). The cellular blue nevus displayed cytoplasmic as well as membranous immunoreactivity (Figure 1d); in contrast, a common blue nevus and a combined nevus, composed of a dermal nevocellular nevus and a blue nevus, did not show any reactivity.

None of the 10 Clark level I lesions in which the MM cells were confined to the epidermis (Figure 1e), and none of the 9 Clark level II lesions that showed a microinvasive component, in the form of single neoplastic cells or small nests of MM cells in the papillary dermis, exhibited ALCAM expression. In contrast, ALCAM expression emerged during melanoma progression through Clark levels III to V (Table 1). Immunoreactivity was observed in 2 of the 13 Clark level III lesions tested. ALCAM expression was membranous and only focally present in the deepest part of the VGP, whereas the RGP was completely negative (Figure 1f). In 13 of the 19 Clark level IV lesions tested, immunoreactivity was observed. Unequivocal membranous staining was observed in 7 of these 13 positive lesions. When both RGP and VGP were present, membranous and/or cytoplasmic immunoreactivity was observed only in the VGP of the tumor, whereas the RGP remained completely negative. The highest percentage of ALCAM-positive tumor cells was observed in the most invasive part of the tumor. One case showed a Clark level IV MM with an adjacent dermal nevocellular nevus; in this case, only the MM showed immunoreactivity. All four Clark level V lesions showed ALCAM expression. Three of these showed unequivocal membranous staining on variable numbers of neoplastic cells in the VGP (Figure 1g). Membranous immunoreactivity in cell clusters always occurred at sites of contacts between neoplastic cells, whereas no immunoreactivity could be detected at the tumor-stroma interface at the periphery of cell clusters (Figure 1h). Comparing the noninvasive melanomas (Clark levels I and II) with the highly invasive melanomas (Clark levels IV and V), expression occurred only in the highly invasive tumors (P < 0.002). The percentage of ALCAM-positive cells in each lesion never exceeded 30% of the total number of melanocytic cells in the primary tumor (Table 1). Based on the Breslow classification, ALCAM expression also correlated with increasing thickness of the primary MMs (Table 1, P < 0.002). Immunoreactivity was observed in less than 10% of the
Figure 1. ALCAM-immunoreactivity in frozen sections of normal skin (a), benign nevi (b–d), primary MM (e–h), and metastatic melanoma (i–k). a: Normal skin. ALCAM is expressed by the inner sheath of the hair follicle, the arrector pili muscle, small nerve fibers, and a subset of cells in the acini of eccrine sweat glands. b: Dermal nevocellular nevus lacking ALCAM immunoreactivity; positivity is restricted to small nerve fibers in between the nevus cells. c: Dermal nevocellular nevus showing weak, granular ALCAM staining in areas of neuroid metaplasia but not in regular nevus cells. d: The nevus cell nests in this cellular blue nevus show both membranous and cytoplasmic ALCAM expression (arrowheads); in addition, a small nerve fiber is stained. e: Superficial spreading MM. RGP lacking ALCAM immunoreactivity. f: Superficial spreading MM in RGP and VGP. Whereas the RGP is negative, intense ALCAM expression occurs in the VGP. g and h: High-power view of neoplastic melanocytes in the VGP of a Clark IV and Clark V MM, respectively. g: ALCAM expression is distinctly membranous. h: ALCAM expression occurs predominantly at sites of contact with adjacent neoplastic cells and not at sites of tumor cell-stroma interaction (arrowheads). i and j: Serial sections of a lymph node metastasis of MM. i: The metastatic MM cells exhibit diffuse expression of ALCAM. j: No immunoreactivity is seen when anti-ALCAM antibody is preabsorbed with an excess of recombinant ALCAM. k: Metastatic MM in a lymph node, showing scattered ALCAM positive neoplastic cells. Three-step avidin-biotin complex technique, counterstained with Harris' hematoxylin; original magnifications, ×88 (a–f, i–k) and ×219 (g, h).
MMs that were thinner than 1.5 mm and in over 70% of the lesions that were thicker than 1.5 mm.

Metastatic lesions showed a decreased incidence in ALCAM immunoreactivity, although this is not significantly different from Clark level V lesions (P > 0.05). Only 7 of the 17 lymph node metastases tested showed ALCAM expression (Table 1, Figure 1i). Membranous immunoreactivity occurred in four of these. In addition to clusters of positive cells, immunoreactivity was observed in scattered neoplastic cells in 4 of the 28 metastatic lesions (Figure 1k).

While in primary MM the number of positive cells rarely exceeded 10%, the overall positivity increased over 50% of all tumor cells in some lymph node metastases. Of 3 ALCAM-negative lymph node metastases, the primary lesions were also available for study; only one of these primary Clark IV MM showed weak cytoplasmic expression in less than 5% of the tumor cells. Six of the 11 cutaneous metastases tested exhibited ALCAM expression in less than 10% of the cells. Of these, two lesions showed unequivocal membranous expression. No statistical differences were observed between ALCAM expression in cutaneous and lymph node metastases (P > 0.05).

All controls were invariably negative and included 1) the replacement of the primary IgG1 antibody by an arbitrary antibody of similar isotype, 2) the use of chromogen alone, and 3) preincubation of anti-ALCAM antibody with an excess of recombinant ALCAM (Figure 1j).

Discussion

Successful metastasis requires an ordered series of sequential steps, including detachment of tumor cells from the primary neoplasm, invasion into and migration through the extracellular matrix, entry into blood and/or lymph vessels, transport along the circulatory system, adhesion to endothelium, extravasation, and outgrowth in a distant organ.13–15 Because correct modulation of adhesive properties is crucial throughout this complex process, correctly timed up- and/or down-regulation of adhesion molecules are considered to be of major importance.16 During the past years, changes in the expression patterns of adhesion molecules have been studied to understand the molecular mechanisms involved in the formation of secondary tumors.17–19

This study is the first to present data with respect to ALCAM expression during melanocytic tumor progression. By using immunohistochemistry on a large series of freshly frozen benign and malignant melanocytic cell lesions, ALCAM expression was observed in the VGP of MM, where it significantly increased with more advanced Clark levels of tumor invasion and correlated with tumor thickness (Table 1). Our data also showed that ALCAM was rarely expressed in benign nevi. Only a cellular blue nevus showed genuine membranous expression of ALCAM. Previous studies have shown that the pigment cells forming cellular blue nevi express various molecules that are normally found on MM.20

The transition from the RGP to the VGP is a biologically and clinically critical step because VGP melanomas have the competence to invade the dermis and subsequently metastasize, whereas RGP melanomas do not have this capacity.21 ALCAM expression occurred exclusively in the VGP and not in the overlying RGP of primary MM, indicating de novo expression of ALCAM in the course of tumor progression and suggesting that ALCAM plays an important role in melanoma cell invasion and melanoma progression. ALCAM expression was observed less frequently in melanoma metastases as compared with ad-

### Table 1. ALCAM Expression in Benign Nevi, Primary Malignant Melanoma, and Melanoma Metastases

<table>
<thead>
<tr>
<th>Positivity *</th>
<th>Benign lesion</th>
<th>MM Clark levels</th>
<th>MM Breslow thickness</th>
<th>Metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-I</td>
<td>C-II</td>
<td>C-III</td>
<td>C-IV</td>
</tr>
<tr>
<td>&gt;50%</td>
<td>dots</td>
<td>dots</td>
<td>dots</td>
<td>dots</td>
</tr>
<tr>
<td>41–50%</td>
<td>dots</td>
<td>dots</td>
<td>dots</td>
<td>dots</td>
</tr>
<tr>
<td>31–40%</td>
<td>dots</td>
<td>dots</td>
<td>dots</td>
<td>dots</td>
</tr>
<tr>
<td>21–30%</td>
<td>dots</td>
<td>dots</td>
<td>dots</td>
<td>dots</td>
</tr>
<tr>
<td>11–20%</td>
<td>dots</td>
<td>dots</td>
<td>dots</td>
<td>dots</td>
</tr>
<tr>
<td>5–10%</td>
<td>dots</td>
<td>dots</td>
<td>dots</td>
<td>dots</td>
</tr>
<tr>
<td>1–5%</td>
<td>dots</td>
<td>dots</td>
<td>dots</td>
<td>dots</td>
</tr>
<tr>
<td>N* /N†</td>
<td>4/38</td>
<td>0/10</td>
<td>0/9</td>
<td>2/13</td>
</tr>
<tr>
<td>%</td>
<td>10.5%</td>
<td>0%</td>
<td>0%</td>
<td>15.4%</td>
</tr>
</tbody>
</table>

*Fraction of positive melanocytic cells in a lesion.
†Number of ALCAM positive lesions (N) per total number of lesions (N) tested.
‡Cellular blue nevus showing membranous and cytoplasmic positivity.
§Weak cytoplasmic positivity in a congenital dermal nevocellular nevus.
¶Cytoplasmic positivity was observed in a congenital dermal nevus and in the areas of neuroid differentiation in a dermal nevus.
●The dermal part of one nevocellular nevus showed membranous staining; its junctional component showed weak cytoplasmic staining.
vanced primary MMs. This finding may be explained by the hypothesis that ALCAM function is indispensable for melanoma cells to invade locally and to gain access to metastatic routes but is dispensable after arrival at the secondary site.

In MM of the human skin, a selective expression profile and association with tumor thickness are not unique. Particularly, some integrins have been shown to play an important role in the invasiveness of various neoplasms.

In MM, the integrin β3 subunit is also associated with tumor thickness and with the ability to invade and metastasize. In analogy to ALCAM, the expression of the β3 subunit of the vitronectin receptor (αvβ3) is exclusively restricted to neoplastic cells in the VGP and in metastatic melanoma. Furthermore, the expression profiles of intercellular adhesion molecule 1 and MUC18 also correlate with vertical tumor thickness.

ALCAM expression was not observed in all neoplastic cells of the VGP (Table 1). In addition to tumor heterogeneity, the focal expression of ALCAM in MMs may be due to its transient nature. T cells and macrophages express ALCAM on activation with phorbol esters and macrophage colony-stimulating factor, respectively. Although a transient ALCAM expression is observed neither in human melanoma cell lines in vitro nor in their derived xenografts (unpublished data), expression of ALCAM on MM cells in vivo may represent a transient phenomenon, related to their activation status. The increase in ALCAM expression during the primary tumor progression and subsequent loss of expression in the melanoma metastasis further favors the concept of a mechanism that can regulate ALCAM expression. The nature of the activating stimulus is currently unknown, but appears to affect single neoplastic cells because neighboring cells are often negative.

This study provides strong evidence that ALCAM expression is a distinguishing feature of the invasive component of primary melanoma and, therefore, a new neoplastic progression marker. The implicit suggestion that ALCAM is involved in mobility and/or growth of subpopulations of cells is also supported by its selective expression in other cell types in the skin, such as Langerhans’ cells, hair follicles, and nerve ends. Future research must therefore be aimed at answering to what extent ALCAM expression would be indispensable for cell mobility and/or growth of these subpopulations.

Acknowledgments
The authors thank Paula Aertsen for excellent technical assistance, Judith Nelissen and Carl Figdor for providing the recombinant ALCAM, and Mikala Egeblad for critical reading of the manuscript.

References
24. Kornberg LJ: Focal adhesion kinase and its potential involvement in cancer invasion and metastasis further favors the concept of a mechanism that can regulate ALCAM expression. The nature of the activating stimulus is currently unknown, but appears to affect single neoplastic cells because neighboring cells are often negative.

References
2. Clark WH Jr, Elder DE, Guerry D, Epstein MN, Greene MH, Van Horn

