Attenuation of Melanoma Invasion by a Secreted Variant of Activated Leukocyte Cell Adhesion Molecule

Jeroen W.J. van Kilsdonk,1 Roel H. Wilting,1 Mieke Bergers,2 Goos N.P. van Muijen,3 Joost Schalkwijk,2 Léon C.L.T. van Kempen,2 and Guido W.M. Swart1

1Department of Biomolecular Chemistry 271, Institute for Molecules and Materials and Nijmegen Centre for Molecular Life Sciences, Faculty of Science, Radboud University Nijmegen; and Departments of Dermatology and Pathology, Radboud University Medical Centre, Nijmegen, the Netherlands

Abstract

Activated leukocyte cell adhesion molecule (ALCAM/CD166/MEMD), a marker of various cancers and mesenchymal stem cells, is involved in melanoma metastasis. We have exploited a secreted NH2-terminal fragment, sALCAM, to test the hypothesis that ALCAM coordinates tissue growth and cell migration. Overexpression of sALCAM in metastatic melanoma cells disturbed clustering of endogenous ALCAM and inhibited activation of matrix metalloproteinase-2 (MMP-2). Exposure of HT1080 fibrosarcoma cells to sALCAM similarly inhibited MMP-2, suggesting a broader effect on ALCAM-positive tumor cells. In contrast to the previously reported, promotive effects of an NH2-terminally truncated, transmembrane variant (ΔN-ALCAM), sALCAM impaired the migratory capacity of transfected cells in vitro, reduced basement membrane penetration in reconstituted human skin equivalents, and diminished metastatic capacity in nude mice. Remarkably, L1 neuronal cell adhesion molecule (L1CAM/CD171), another progression marker of several cancers including melanoma, was suppressed upon sALCAM overexpression but was up-regulated by ΔN-ALCAM. The partially overlapping and opposite effects induced by alternative strategies targeting ALCAM functions collectively attribute an integrative role to ALCAM in orchestrating cell adhesion, growth, invasion, and proteolysis in the tumor tissue microenvironment and disclose a therapeutic potential for sALCAM.

[Cancer Res 2008;68(10):3671–9]

Introduction

Cancer is frequently accompanied by aberrant distributions and/or functions of cell adhesion molecules, e.g., involving members of the cadherin and/or immunoglobulin families (1–3). Aberrant expression profiles of activated leukocyte cell adhesion molecule (ALCAM/MEMD/CD166) were reported in a series of different cancer tissues and cells (e.g., melanoma, prostate carcinoma, breast cancer, esophageal squamous cell carcinoma, colorectal carcinoma, and bladder cancer; refs. 4–9). Additionally, random phage display searches identified ALCAM as a readily accessible, therapeutic target on different cancer cells (10, 11). ALCAM is a member of the small VVC2C2C2 subgroup of the immunoglobulin superfamily, which also includes MUC18/MelCAM/CD146 and B-CAM/Lutheran/CD239. ALCAM shows a characteristic, temporal, and spatial distribution in development of a wide variety of tissues and cells in health and disease. Besides cancer, ALCAM is involved in embryogenesis, neurogenesis, angiogenesis, hematopoiesis, immune response, and it is applied as a marker of pluripotent mesenchymal stem cells (for reviews, see refs. 12, 13).

In general, dynamic modulation of cell adhesion is crucial for tissue architecture, growth, and migration. It tunes cellular responses and microenvironmental signals and maintains the proper embedding of cells in a tissue. To address the pleiotropic roles of ALCAM in cancer dynamics, we have proposed a working model for its homophilic cell adhesion function based on structure-function analyses (Fig. 1A; refs. 14, 15). Briefly, oligomerization on the cell surface and intercellular receptor–ligand binding would synergistically promote ALCAM recruitment and network formation in regions of cell-cell contact. The dual functions are provided by an oligomerization module, which consists of the membrane-proximal, C2-type immunoglobulin domains (D3–D5), and a ligand-binding module, which consists of the membrane-distal, V-type immunoglobulin domains (D1–D2). ALCAM clustering may be controlled by unconventional anchoring of its short cytoplasmic tail (32 amino acids) to the actin cytoskeleton (16, 17). Cognate ALCAM properties are disturbed by a deletion mutant, ΔN-ALCAM, which conserves the oligomerization properties (domains D3–D5) but lacks the ligand-receptor binding function (D1–D2). Ectopic expression of ΔN-ALCAM interferes with intercellular ALCAM network formation (Fig. 1B). Overexpression of ΔN-ALCAM in metastatic melanoma cells not only interfered with endogenous ALCAM but also aggravated the invasive cell behavior (18). Unexpectedly, increase and acceleration of spontaneous metastasis in nude mice were accompanied by the near-complete inhibition of the activation cascade for matrix metalloproteinase-2 (MMP-2; ref. 19). The overall phenotype induced by ΔN-ALCAM was remarkable because it apparently uncoupled proteolysis from invasiveness. The association of these properties is commonly believed to favor tumor growth and metastasis. Therefore, the results suggested that the proper integration of oligomerization and ligand receptor–binding properties of ALCAM could be pivotal to coordinate tumor tissue development and invasion.

Our previous experiments with ΔN-ALCAM primarily targeted the oligomerization module. The present study aimed at supplementing this strategy by targeting the ligand binding module via overexpression of a secreted (s)ALCAM variant (Fig. 1C). The sALCAM variant, which apparently impaired cell-cell interactions, was recently isolated as an alternative, shortened ALCAM transcript in endothelial cells (20). This transcript comprised the first three exons and only encoded the NH2-terminal signal sequence.
plus immunoglobulin domain D1. Immunoglobulin domain D1 was required for ligand binding (14). Exposure of endothelial cells to recombinant sALCAM increased cell migration and decreased capillary tube formation (20). We thus anticipated that transfection of sALCAM into metastatic melanoma cells would influence cell clustering by challenging ligand-receptor binding and intercellular network formation of endogenous ALCAM (cf. Fig. 1C) and, as a consequence, affect the coordination of proteolysis, tumor growth, and metastasis (cf. ref. 19).

Whereas the ectopic ΔN-ALCAM mainly aggravated the invasive behavior of the previously metastatic melanoma cells, the present series of experiments revealed that overexpression of sALCAM primarily attenuated the invasive properties of the targeted cells. Furthermore, we found that disturbance of ALCAM function had a major effect on neuronal L1 cell adhesion molecule (L1CAM/L1), which is involved in lateral ALCAM oligomerization thereby increasing the avidity of homophilic ligand binding mediated by the ligand binding module (ib) consisting of D1 and D2. B, model for amino-terminally truncated ALCAM (ΔN-ALCAM). Ectopic expression of ΔN-ALCAM comprising domains D3 to D5 interferes with intercellular ALCAM network formation. C, model for soluble ALCAM (sALCAM). sALCAM refers to secreted domain D1, which is involved in ligand binding. sALCAM presumably blocks homophilic receptor ligand binding.

Materials and Methods

Cell culture. Adherent melanoma cells (BLM) and HT1080 fibrosarcoma cells were maintained as monolayers in DMEM (Invitrogen) supplemented with 10% FCS, 4 mmol/L L-glutamine, and 2 mmol/L pyruvate. BLM cells with amino-terminally truncated ALCAM (BLM/ΔN-ALCAM) were cultured as described previously (14, 28). If indicated, concanavalin A (ConA; Sigma-Aldrich) was added to serum-free medium (20 μg/mL). All cells were grown to desired densities or split before confluence was reached. Three-dimensional collagen cultures were prepared as described previously (19). Frequent testing certified that cells were free of Mycoplasma contamination.

Generation of BLM/sALCAM cells. For the generation of BLM/sALCAM cells, the vector sALCAM-p3XFLAG was transfected into BLM cells using Sanmix (Synvolux Therapeutics). A mixture of 20 μL Sanmix and 80 μL HBS was made. Circular DNA (1.7 μg) was added to 100 μL HBS in a separate tube. After 5 min of incubation at room temperature, both mixes were combined and supplemented with 800 μL medium. This DNA/Sanmix mixture was added directly to a 40% confluent monolayer. Cells were incubated at 37°C and 2 mL medium was added after 3 h. For stable transfection, the medium was supplemented with 0.5 mg/mL G418 (Life Technologies/Invitrogen) 48 h after transfection. Single, neomycin-resistant cells were sorted by flow cytometry and expanded in medium containing 0.5 mg/mL G418.

In vitro wound-healing assay. Wound-healing assays were done on confluent monolayers inflicted with a “wound” made by a pipette tip. Detached cells were removed by washing with medium (37°C). Cell migration into the scratch was monitored at various time points starting from 2 h after wounding to avoid effects of cell spreading. Images were analyzed using CorelDraw 10 (Corel Corporation). Gap width was measured at six positions per inflicted wound in six independent confluent cultures. To prevent interference of cell doubling, the experiment did not exceed the doubling time of the cells tested (18 h).

Reverse transcription-PCR and quantitative real-time PCR. RNA was isolated using the RNeasy kit (Qiagen) and after removal of genomic DNA (DNase I, Invitrogen) was used as cDNA synthesis template using Superscript III reverse transcriptase (Invitrogen). cDNA was diluted 1/50 and stored at −20°C.

 Primer sequences are given in Supplementary Table S1. All primers were synthesized by Isogen Lifesciences. cDNA levels were determined using SYBR green fluorescence with the ABI-Prism 7000 (Applied Biosystems). Expression levels of three individual experiments were evaluated as the number of cycle difference from glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Significant changes from control samples were determined using Student’s t test. P ≤ 0.05 was considered significant.

Western blotting and gelatin zymography. Cell extracts were prepared and Western blotted as described previously (19). Serum-free media were precipitated using 2-propanol at −70°C for 20 min followed by 90 min at −20°C; precipitates were taken up in reducing sample buffer. After 5 min boiling, samples of medium precipitates or cell lysates (20 μg protein) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). Anti–FLAG M2 antibody (1:1,000; Stratagene) was used to detect FLAG-tagged proteins. The monoclonal antibodies AZN-L50 and L1-I1A were used to detect ALCAM and L1CAM, respectively. Bound primary antibodies were visualized by appropriate horseradish peroxidase–coupled secondary antibodies and enhanced chemiluminescence (ECL plus, Amersham Biosciences). Analysis of nude mouse xenografts was performed on four nonnecrotic 10–mm (diameter) frozen tissue sections solubilized in 15 μL of reducing sample buffer, of which 5 μL samples were loaded on gel. Gelatin zymography was described previously (19).

Confocal laser scanning microscopy. Cells were grown on glass coverslips and, after PBS washing, fixed in 4% paraformaldehyde in PBS (15 min room temperature). After thorough washing with PBS (3×), cells were rendered permeable in PBS containing 0.1% Triton X-100 for 1 min. To block unspecified binding of the antibody, cells were incubated in 1% bovine serum albumin (BSA) in PBS for 30 min. Cells were stained overnight using 3 μg/μL AZN-L50 (in 1% BSA/PBS) at 4°C and subsequently incubated with...
FITC-conjugated rabbit–anti-mouse (I:1,000 in PBS; Dako) for 1 h at room temperature. Unbound antibodies were washed off using PBS. Finally, the coverslips were mounted in Mowiol and sealed with nail polish.

**De-epidermized dermis.** Skin samples were obtained from donors who underwent abdominal skin corrections by plastic surgery. Separation of epidermis and dermis was catalyzed by a 5- to 10-min incubation in PBS at 56°C. The dermis was incubated for 4 wk in PBS containing antibiotics at 37°C. Standardized de-epidermized dermis (DED) pieces were obtained as 8-mm punch biopsies.

**Generation of human skin equivalents.** DEDs (basement membrane side up) were placed in transwells and seeded on top with 10⁵ keratinocytes and 10⁴ melanoma cells in DED medium (adapted from ref. 29) supplemented with 5% defined bovine calf serum (Hyclone). DED medium consisted of DMEM/Ham’s F12 (2:1) medium, L-glutamine (4 mmol/L), penicillin/streptomycin (50 IU/mL), ascorbic acid (50 μg/mL), insulin (0.2 μg/mL), hydrocortisone (1 μmol/L), triiodothyronine (1.36 ng/mL), and cholera toxin (0.1 nmol/L). The cells were kept in submerged culture for 3 d. Then, the reconstructs were lifted to the air-liquid interface and kept for 10 d in DED medium supplemented with t-serine (1 mg/mL), t-carnitine (2 μg/mL), BSA lipid mix palmitic acid (25 μmol/L), arachidonic acid (7 μmol/L), linoleic acid (15 μmol/L), vitamin E (0.4 μg/mL in 20 mmol/L BSA in PBS), and epidermal growth factor (5 ng/mL). After harvesting, the skin equivalents were formalin fixed and embedded in paraffin.

**Immunohistochemistry.** Sections (6 μm) were deparaffinized in ethanol and rehydrated for immunohistochemical analysis of heparan sulfate proteoglycan antibody JM-403 (1:1,000; ref. 30) and calcyclin (1:500; ref. 31). After incubation with primary antibodies, sections were stained with avidin-biotin-peroxidase complex system (Vectastain elite ABC kit; Vector Laboratories, Inc.). To visualize antibody binding, AEC staining (Calbiochem) was performed. Counterstaining was performed with Mayer’s hematoxylin (Sigma). Finally, sections were washed in tap water, dried, and mounted in glycerol gelatin (Sigma). In addition, we performed H&E staining on serial sections.

**In vivo tumor growth and metastasis.** Male BALB/c athymic nude mice (nu/nu) were bred and kept under specific pathogen-free conditions.
Tumor cells were harvested using trypsin/EDTA, washed, and suspended in PBS (2 \times 10^7/mL). Mice, 7 wk old, were s.c. inoculated with 100 \times 10^6 cells suspension. Every 3 d, mice were inspected and local tumor size was measured. At the indicated times, mice were sacrificed and tumors were dissected, snap frozen in liquid nitrogen, and stored at \(-70^\circ C\). Non-necrotic tumor samples were analyzed by Western blotting. Lungs were prepared with Tissue-tek OCT compound (Sakura), fixed in formalin, and embedded in paraffin for microscopic examination of H&E-stained sections. Lung metastases were counted at multiple sections (three to five) within the lungs to compare equal surface areas. Sizes of the metastatic lesions were measured as cells per single lesion.

Results

Expression of sALCAM. The recent isolation of an alternative transcript encoding a secreted sALCAM variant in endothelial cells hinted at a novel regulatory mechanism of ALCAM (20). Therefore, we determined if sALCAM transcripts were present in the human melanoma cell line BLM. Semiquantitative PCR assays revealed positive signals for GAPDH and ALCAM, whereas no sALCAM signal could be observed (not shown). In agreement with previous Northern blot analyses (28), we again concluded that BLM cells expressed neither alternative ALCAM transcripts nor any related secreted ALCAM isoforms.

Then, we transfected sALCAM expression constructs into BLM cells to check the effects on cognate ALCAM. A series of stable cell clones was generated expressing and secreting sALCAM, ranging from low (BLM/sA<sub>low</sub>), medium (BLM/sA<sub>med</sub>), to high secretion (BLM/sA<sub>high</sub>). Using the COOH-terminally attached FLAG-epitope tag, sALCAM was detected in cell lysates (Fig. 2A, left) and conditioned culture medium of BLM/sALCAM cells (Fig. 2A, right), confirming protein expression and secretion. The double bands on the Western blot were due to two N-linked glycosylation sites in sALCAM (20). It was noticed in the course of the experiments that BLM/sA<sub>high</sub> clones tended to completely lose sALCAM expression. The reverted phenotype was always comparable with parental BLM (results not shown). sALCAM expression in BLM/sA<sub>high</sub> remains constant and BLM/sALCAM generally refers to BLM/sA<sub>med</sub> cells in the following sections.

Figure 3. sALCAM expression prevents MMP-2 activation. A, zymography of medium conditioned by BLM, HT1080, and/or BLM/sALCAM. Cells were grown as superconfluent monolayers (>2.5 \times 10^5/cm²) and in three-dimensional type 1 collagen cultures (7.5 \times 10^5 per gel). After 24 h of incubation, all samples taken from monolayer cultures are negative for the 59 kDa active form of MMP-2 (act MMP). BLM cells, and to a lower extent sA<sub>low</sub>, are capable of activating MMP-2 when grown in collagen. The effect of sALCAM on HT1080 was assessed by preparing separate collagen gels embedded with either BLM/sALCAM or HT1080 (5.0 \times 10^6 cells each); after 24 h, the two gels were transferred to one well and incubation was continued with fresh serum-free medium for another 24 h before conditioned medium was collected from the combined culture (right). Note that physical contact between HT1080 and BLM/sALCAM was avoided in this setup. B, quantitative PCR analysis of MT1-MMP, TIMP-2, and MMP-2 mRNA levels in BLM and BLM/sALCAM cells. Cells were cultured as monolayers at high cell density (>2.5 \times 10^5/cm²). mRNA levels of BLM cells (white columns) were compared with messenger levels of BLM/sALCAM cells (black columns). Expression levels of BLM cells were set to 100% for each gene. sALCAM expression significantly reduces the expression of MT1-MMP and MMP-2. TIMP-2 levels are unaffected by sALCAM. Experiments were performed in triplicates. *, P ≤ 0.05 significant difference (Student's t test). C, BLM, BLM/ΔN-ALCAM, and BLM/sALCAM cells were cultured as monolayers in a 24-well plate at low density (1.0 \times 10^5). After overnight incubation, cells were treated without or with ConA for 24 h. Conditioned media were harvested and subjected to gelatin zymography. Only BLM activates MMP-2 when ConA is added. D, mock and siRNA (against ALCAM) transfected cells were treated with or without ConA. Knockdown of ALCAM cannot block the ConA mediated activation of MMP-2.
sALCAM effects on ALCAM clustering and cell migration. Previous confocal experiments showed strong fluorescent bands reflecting recruitment of ALCAM and apparent network formation in regions of cell-cell contact (Fig. 2B, BLM, arrows; cf. ref. 28). Upon overexpression of sALCAM, the banding pattern was lost and the transfected cells maintained an even ALCAM distribution all along the plasma membrane despite direct cell-cell contacts (Fig. 2B, BLM/sALCAM, arrowheads). This suggested that sALCAM impaired focal recruitment of ALCAM clusters along the plasma membrane and, as a consequence, prevented extended ALCAM network formation. Cell morphology was not significantly affected by sALCAM.

The disruption of the ALCAM network by ΔN-ALCAM resulted in increased cell mobility compared with BLM in a wound-healing assay in vitro (Fig. 2C; cf. ref. 18). BLM/ΔN-ALCAM cells rapidly migrated into the gap as scattered cells. By contrast, BLM/sALCAM cells were retarded in gap closure relative to control cells (Fig. 2C; 16% gap closure for BLM/sALCAM in 8.5 h compared with 32% for BLM). Thus, interfering with ALCAM-ALCAM interactions by sALCAM decreased cell mobility in vitro.

sALCAM and the activation cascade of MMP-2. The activation cascade of MMP-2 involves the assembly of a ternary complex of MT1-MMP, tissue inhibitor of metalloproteinase-2 (TIMP-2), and pro–MMP-2 (32, 33). We previously reported that efficient triggering of this activation cascade equally depended on sufficient cell-matrix and cell-cell interactions and required functional ALCAM (19). To investigate putative sALCAM effects on MMP-2 activation, a representative series of BLM/sALCAM cells were grown to high cell densities as two-dimensional monolayer and three-dimensional collagen cultures. Zymography revealed one single band of 72 kDa in the conditioned medium from all two-dimensional monolayer cultures, representing pro–MMP-2 (Fig. 3A, monolayer; cf. refs. 19, 34). Furthermore, the amounts of secreted pro–MMP-2 diminished with increasing levels of sALCAM. BLM cells grown in collagen gels processed pro–MMP-2 via an intermediate (62 kDa) to active MMP-2 (59 kDa; Fig. 3A, collagen matrix). BLM clones secreting sALCAM at intermediate and high levels showed no activation of MMP-2 at all, whereas secretion of sALCAM at low levels reduced the conversion of pro–MMP-2 to active MMP-2 compared with control cells (Fig. 3A, collagen matrix). Therefore, the apparent disruption of the ALCAM network by sALCAM prevented MMP-2 activation. This result was similar to the effects induced by ΔN-ALCAM and ALCAM depletion (19).

To investigate whether the induced effects would be more generally applicable, collagen gels of human fibrosarcoma HT1080 and BLM/sALCAM cells were separately prepared and transferred to one culture well after 24 hours with the aim to evaluate the effects of sALCAM on HT1080. Physical contacts between HT1080 and BLM/sALCAM were avoided in this experimental setup. Zymography analysis of the biculture conditioned medium revealed that sALCAM also decreased the levels and activation of MMP-2 in HT1080 (Fig. 3A, HT1080). The presence of sALCAM also correlated with a reduced amount of MMP-9 (this effect was not further analyzed). The analysis of conditioned medium from control

![Figure 4](image-url)

Figure 4. sALCAM affects the invasive potential of BLM cells in reconstructed skin. The invasive potential of BLM cells expressing sALCAM was tested in human reconstructed skin. H&E, heparan sulfate, and calcyclin staining were performed on the skin constructs. A, sAhigh cells showed no invasion into the dermis. sAMed cells invade the dermis through the basement membrane as single cells; however, many cells remain in the reconstructed epidermis. BLM/sALCAM cells with low expression of sALCAM (sAlow) are aggressively invading the dermis, compared with sAMed in which few cells remain in the reconstructed epidermis; B, the presence of basement membranes is shown by heparan sulfate staining. C, tumor cells are identified with calcyclin staining. D, melanoma cells accumulate on top of basement membrane and dermal structures in the absence of keratinocytes and epidermis.
Table 1. Metastatic potential of BLM/sALCAM cells in nude mice

<table>
<thead>
<tr>
<th>Cells</th>
<th>Incubation (d)</th>
<th>Primary tumor weight (g)</th>
<th>Animals with lung metastasis</th>
<th>No. metastasis in lung sections*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>n/total</td>
<td>Fraction of total (%)</td>
</tr>
<tr>
<td>BLM/control</td>
<td>24</td>
<td>&lt;4</td>
<td>3/5</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>&gt;4</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>BLM/sALCAM</td>
<td>34</td>
<td>&lt;4</td>
<td>2/7</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>&gt;4</td>
<td>5/8</td>
<td>63</td>
</tr>
</tbody>
</table>

NOTE: The metastatic potential of BLM cells expressing sALCAM was tested in male nude BalB/c mice. [The average number of metastasized cells in the lungs of BLM/sALCAM mice at day 34 is significantly lower compared with control animals (P ≤ 0.05, Mann-Whitney test). BLM/control cells (cf. Fig 2) and BLM/sALCAM cells were s.c. inoculated. All mice showed tumor take and local tumor growth was measured every 3 d. The mice were kept for 24 or 34 d to vary primary tumor sizes. The average weight of all 25 tumors was 4 g; all tumors taken at day 24 were smaller than 4 g, and all tumors taken at day 34 were bigger than 4 g. The table shows the numbers and percentages of animals with lung metastasis. Furthermore, lung metastasis is measured as total numbers and as average number per injected animal.

*A difference of borderline significance (P = 0.028, t test) was found between the number of metastases per mouse at day 34 after inoculation.

cocultures of HT1080 and BLM showed that MMP levels essentially reflected the addition of separate contributions (not shown). The influences of sALCAM on native HT1080 reassured that the effects observed in BLM/sALCAM were indeed due to the secreted protein and not to potential side effects of overexpression.

To check for upstream changes in the cascade, we applied quantitative PCR and found significant decreases in mRNA levels of both MT1-MMP and MMP-2 upon overexpression of sALCAM, whereas TIMP-2 levels did not significantly change (Fig. 3B). The reduced mRNA levels of MMP-2 corresponded with the zymography results (Fig. 3A). Further assessing the deficient conversion of pro–MMP-2 to the active enzyme, we used ConA to test the involvement of MT1-MMP. ConA potently promoted activation of MMP-2 in a MT1-MMP–dependent manner and bypassed the need of cell–matrix interactions (35–38). The addition of ConA to BLM cells in monolayer indeed triggered MMP-2 activation (Fig. 3C) and, furthermore, this induction also bypassed the need for cell-cell contacts (results not shown). BLM cells depleted for ALCAM by small interfering RNA (siRNA) treatment did not activate MMP-2 when grown in collagen (19). Addition of ConA to monolayer cultures of ALCAM-depleted BLM cells, however, induced MMP-2 activation (Fig. 3D). In contrast, the triggering by ConA was abolished in BLM/sALCAM and in BLM/DN-ALCAM cultures (Fig. 3C). Therefore, physiologic activation of the MMP-2 cascade depended on ALCAM. The pharmacologic bypass provided by ConA relieved this dependence but was impaired by the presence of defective ALCAM.

sALCAM and melanoma growth in reconstituted human skin equivalents. After the encouraging observations of reduced cell mobility and inhibition of MMP-2 processing, we were interested to investigate the invasive capacity of BLM/sALCAM cells in reconstituted skin equivalents assembled on human DED. The histologic analysis of the reconstituted skin equivalents revealed an inverse relation between sALCAM overexpression levels and invasive potential of different BLM/sALCAM cells (i.e., sAlow cells were most invasive; sAhigh cells were least invasive; Fig. 4A). The presence of the basement membrane was confirmed by heparan sulfate (Fig. 4B), laminin, and collagen IV (data not shown) staining. Melanoma cells were identified by calcyclin staining (Fig. 4C, arrowheads). In the absence of keratinocytes, all melanoma cells, with or without sALCAM expression, accumulated on top of DED and did not penetrate the basement membrane structures (Fig. 4D). BLM/sAlow cells abundantly invaded the dermal structures as single cells; the number of invasive BLM/sAmed cells was reduced relative to BLM/sAhigh; BLM/sAmed cells did not detectably invade into DED (Fig. 4A). These findings indicated that the overexpression of sALCAM inhibited the invasive potential of BLM.

sALCAM and spontaneous lung metastasis in a xenograft mouse model. To extrapolate the apparent, invasion-suppressing role of sALCAM to an in vivo metastasis model, series of nude mice were s.c. inoculated with BLM/sALCAM cells. Tumor take was detected in all animals after 14 days and the xenograft growth was recorded twice a week. Mice were sacrificed after 24 or 34 days to vary primary xenograft sizes. In time, average tumor volumes of BLM/sALCAM did not differ from those of BLM/control, mimicking any proliferation bias (Supplementary Fig. S1). After sacrificing the mice, primary tumors were weighed and lungs were collected for microscopic analysis. The average weight of all primary tumors was ~4 g; all tumors harvested at day 24 were less than 4 g, and all tumors collected at day 34 exceeded 4 g (Table 1). Although primary tumor weights were comparable, BLM/control cells displayed more frequent lung metastasis than BLM/sALCAM cells; this is true for both groups with small and large tumor sizes (Table 1: <4 g, 60% versus 29%, respectively; and >4 g, 100% versus 63%, respectively). BLM/sALCAM cells were not only attenuated in metastasis frequency but also the number of metastatic lesions was smaller. In the group of large tumors (>4 g), BLM/control induced almost thrice as many metastatic lesions on average compared with BLM/sALCAM (8 versus 2.75 lesions per injected animal, respectively; Table 1). In line with the previous in vitro results, the overexpression of sALCAM thus attenuated the in vivo metastatic phenotype of BLM.

ALCAM and expression of LICAM/CD171. In recent years, the neuronal cell adhesion molecule LICAM was repeatedly reported as a marker of carcinoma and melanoma (22, 24, 25, 39). Additionally, affinity chromatography experiments with avian cells detected an interaction between the chicken variants of ALCAM and LICAM (40). Therefore, it was worthwhile to determine the levels of LICAM expression in our series of BLM cells...
overexpressing mutant ALCAM variants and to see if L1CAM could contribute to the significantly changed, invasive properties. Western blot analysis indeed revealed considerable L1CAM protein levels in BLM cell extracts (Fig. 5A). Remarkably, sALCAM overexpression almost completely suppressed L1CAM, whereas ΔN-ALCAM overexpression increased the levels of L1CAM (Fig. 5A). Subsequently, we also analyzed tumor extracts from nude mice experiments for the presence of L1CAM. Western blotting again detected relative decreases of L1CAM in extracted BLM/sALCAM xenografts and increases in BLM/ΔN-ALCAM (Fig. 5B). We also checked the xenograft levels of sALCAM by Western blotting and confirmed its stable overexpression (Fig. 5B, bottom).

In conclusion, alternative targeting of ALCAM had a major effect on L1CAM expression both in vitro and in vivo, suggesting orchestrated roles of ALCAM and L1CAM in tumor development.

Discussion

Tissue development is a highly complex process in which dynamic growth of cells and stroma generates and maintains tissue architecture. Although oncogenesis may hijack developmental pathways, tumor growth is counterproductive and frequently results in metastasis and host killing. ALCAM is involved in development and oncology, but its mechanism of action is still elusive. Using a melanoma model, this study addresses the hypothesis that the modular structure of ALCAM (cf. Fig. 1) integrates diverse functions involved in coordination of local growth and metastasis. The secreted variant sALCAM, which is anticipated to target the ligand-binding module, prevents the apparent focal recruitment of ALCAM clusters and interrupts extended, intercellular ALCAM network formation. Furthermore, sALCAM impairs more complex cellular responses (e.g., MMP-2 activation, L1-CAM expression, cell migration, invasion, and metastasis). The similar inhibition of the MMP-2 activation cascade in HT1080 fibrosarcoma cells indicates that the sALCAM effects may be more generally applicable to the spectrum of ALCAM-positive tumor cells.

The present set of experiments supplements previous characterizations of cellular properties induced by overexpression of ΔN-ALCAM (cf. Fig. 1B). In the latter case, ALCAM malfunction also inhibits MMP-2 activation but nevertheless provides a gateway to melanoma metastasis, thereby uncoupling proteolysis from invasiveness (18, 19). The phenotypes induced by either sALCAM or ΔN-ALCAM thus display both overlapping and opposite cellular properties and cannot be accommodated by a plain role of ALCAM in cell-cell interactions. Closer inspection discloses that the pleiotropic effects are well compatible with a networking role of ALCAM that integrates complex, microenvironmental signaling and invasive growth dynamics.

ALCAM and control of MMP-2 activation. An effective networking role in response to increasing cell clustering seems dependent on ALCAM integrity. In agreement with this, the defects caused by sALCAM inhibit the MMP-2 activation cascade similar to ΔN-ALCAM (Fig. 3; ref. 19). Both strategies diminish expression of the genes encoding the proteases (MMP2, MMP14) without significant changes in TIMP2 levels and thus affect the balance and assembly of MT1-MMP, TIMP2, and pro–MMP-2 to form a ternary complex, which is an intermediate in MMP-2 activation. Furthermore, ALCAM defects prevent the stimulatory actions of ConA, which usually serves as an efficient trigger of the cascade (35–38). ALCAM malfunction therefore introduces a permanent hurdle for ConA effects. By contrast, ConA relieves the inhibition of MMP-2 in the absence of ALCAM after its depletion by siRNA treatment (Fig. 3). Notably, ALCAM depletion is not accompanied by any changes in the levels of MMP2 or MMP14 (19). These observations support the interpretation that ALCAM inserts a conditional control switch in the signaling pathways regulating MMP-2 activation. Overexpression of sALCAM or ΔN-ALCAM aborts the prevailing ConA activation and indicates that the switch is blocked in a disconnected state due to a defective networking role of ALCAM.

ALCAM and control of cell migration and metastasis. In contrast to MMP-2 activation, which apparently requires formation of an integral ALCAM network, the migratory and metastatic

Figure 5. ALCAM function regulates the expression of L1CAM. A, BLM, BLM/sALCAM, and BLM/ΔN-ALCAM cells were cultured as monolayers; cell lysates were subjected to Western blot analysis using a monoclonal antibody against L1CAM (L1-11A). BLM and BLM/ΔN-ALCAM cells are positive for L1CAM in contrast to BLM/sALCAM cells. Both full-length and plasmin-cleaved L1 are detected. B, after s.c. injection into nude mice with different cell lines (BLM, BLM/ΔN-ALCAM, and BLM/sALCAM), frozen nonnecrotic tumor tissues were analyzed by Western blotting and stained for L1CAM and sALCAM with an anti-FLAG antibody. Samples were extracted from independent tumors (three samples for control and sALCAM tumors; two samples for BLM/ΔN-ALCAM tumors). As confirmed by anti-FLAG staining, the sALCAM-positive tumors have the lowest amount of L1CAM. Tumor samples did not contain the (proteolytic) L1 fragment generated by plasmin cleavage (loading control is given in Supplementary Fig. S2).
properties of BLM are differentially dependent on the dissected oligomerizing or ligand-receptor binding modules of ALCAM. Overexpression of sALCAM inhibits ALCAM clustering in line with a deficiency to sustain oligomer recruitment into an extended network (Figs. 1C and 2B). As a consequence, the generation of downstream signals that depend on ALCAM integrity is not sufficient to promote proteolysis, cell migration, and metastasis. Overexpression of ΔN-ALCAM interrupts the integrative function too (Fig. 1B) and impairs proteolysis (19). Nevertheless, the merely increased number of transmembrane oligomer clusters could overall mimic network formation to some extent and thus provide bypasses, which suffice for partial generation of downstream signals and sustain cell migration and metastasis (18). It is noteworthy in this regard that the apparent networking capacity of ALCAM could confer properties supplementary or even dominant to a plain cell adhesion function.

**ALCAM and cell adhesion.** The profile of LICAM in our series of BLM cells directly correlates with the invasive potential and adds a striking feature to the partially dissected and integrated functions of ALCAM. Several reports have identified LICAM as a melanoma progression marker (21–27). Respective overexpression or suppression of LICAM alternatively enhanced or diminished invasiveness (21). Moreover, LICAM can be released into the microenvironment via ectodomain cleavage from cells, catalyzed by a disintegrin and metalloprotease (ADAM) 10 and ADAM17 (41), or via shedding of microvesicles (42). Interestingly, ALCAM may also be shed by ADAM-17, resulting in a soluble form comprising most of the extracellular structures (43). Although the potential of LICAM to serve as a therapeutic target is already explored (44), random phage display strategies have independently identified ALCAM as a novel cell surface target of different cancers with a readily applicable potential for intracellular delivery of a toxic payload (10, 11). Abrupt expression of ALCAM and LICAM may thus synergize and favor tumor progression and metastasis. Furthermore, cell surface metabolism of ALCAM and LICAM may significantly affect dynamic signaling processes, proteolysis, and vesicle turnover at the plasma membrane.

**Functions and nature of ALCAM network formation.** The remarkable cellular responses disclosed in the present and previous sets of experiments raise urgent questions on how ALCAM functions would favor invasive growth processes. The observation of ALCAM expression in human blastocysts and endometrial epithelium (45) suggested that ALCAM could be involved in fetal implantation into the uterine wall. Masedunskas et al. (46) showed that the passage of monocytes through an endothelial monolayer correlated with ALCAM levels in the monocytes and with endothelial ALCAM localized to the lateral plasma membrane. Soluble ALCAM variants and anti-ALCAM antibodies inhibited the trans-endothelial migration of monocytes but did not affect their migration or adhesion to endothelium. Ectopic ALCAM overexpression generally promoted cell-cell interactions (14, 20), whereas soluble, recombinant ALCAM variants attenuated cell-cell interactions (refs. 20, 46; this report). The affinity of ALCAM-ALCAM interactions seemed relatively low in solution (K_D = 29-48 μmol/L; ref. 47). The weakness of homophilic interactions was consistent with the apparent lack of anti-FLAG-tag antibodies to detect the binding of monovalent recombinant sALCAM to cells by flow cytometry and immunofluorescence (results not shown). Formation of a transcellular ALCAM network would thus be characterized by a multitude of weak interactions with fast on and off kinetics. Progressive ALCAM clustering could promote the sequestration or tethering of additional components (e.g., MT1-MMP, LICAM) into this network. Besides, the dependence of robust cell-cell binding on focal ALCAM avidity could be compatible with the inherent plasticity and dynamics required for transmigration of cells (15).

sALCAM, an inhibitory device for metastasis. The experimental evidence presented and discussed above shows divergent biological signals and responses associated with partially dissected and integrated ALCAM functions in BLM and HT1080 cells. Notably, mimicry of limited or enhanced ALCAM recruitment, as induced by respective overexpression of sALCAM or ΔN-ALCAM, directly correlates with attenuated or enhanced metastatic capacities. Whereas many cells commonly express high-affinity, cadherin-type cell adhesion molecules, BLM cells are apparently devoid of cadherins (48). This peculiarity may constitute BLM as a prototype for characterizing relative low-affinity cell adhesion interactions, as mediated by ALCAM. Effective robustness and plasticity of malignant tumor cells presumably require coordinate processing of all microenvironmental signals to transform the input of complex information into uniform cellular interpretations that spatially and temporally control melanoma growth dynamics and metastasis (cf. ref. 49). The available experimental evidence is consistent with the hypothesis that ALCAM activity contributes to a novel processor and integrator function that serially collects and connects multiple upstream, microenvironmental signals, and simultaneously inserts a switch that controls downstream responses. Interestingly, the inhibitory properties of sALCAM interfere with these functions and suggest that sALCAM-like devices with improved binding affinities and blocking effects may have a therapeutic potential.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

Received 10/5/2007; revised 2/29/2008; accepted 3/1/2008.

**Grant support:** Dutch Cancer Society grant KUN-2002-2757.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Rob Woestenak for excellent technical support with flow cytometry cell sorting; Ronald Schildkönig for the HT1080 experiments; Ine Mamor-Cornelissen for the animal experiments; Dr. R. Torensma (Department of Tumor Immunology, Radboud University Medical Centre, Nijmegen, the Netherlands) for the gift of AZN-L5D; Dr. P. Allevot (Tumor Immunology Programme, German Cancer Research Center, Heidelberg, Germany) for the gift of L1-11A antibody; and Thomas Quertermous (Division of Cardiovascular Medicine, Stanford University, Stanford, CA) for the gift of sALCAM-p3XFLAG.

**References**


Attenuation of Melanoma Invasion by a Secreted Variant of Activated Leukocyte Cell Adhesion Molecule

Jeroen W.J. van Kilsdonk, Roel H. Wilting, Mieke Bergers, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/68/10/3671

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2008/05/12/68.10.3671.DC1

Cited articles
This article cites 49 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/68/10/3671.full#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/68/10/3671.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/68/10/3671.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC)
Rightslink site.