Activated Leukocyte Cell Adhesion Molecule (ALCAM/CD166/MEMD), a Novel Actor in Invasive Growth, Controls Matrix Metalloproteinase Activity

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Abstract

Activated leukocyte cell adhesion molecule (ALCAM/CD166/MEMD) could function as a cell surface sensor for cell density, controlling the transition between local cell proliferation and tissue invasion in melanoma progression. We have tested the hypothesis that progressive cell clustering controls the proteolytic cascade for activation of gelatinase A/matrix metalloproteinase-2 (MMP-2), which involves formation of an intermediate ternary complex of membrane type 1 MMP (MT1-MMP/MMP-14), tissue inhibitor of metalloproteinase-2 (TIMP-2), and pro–MMP-2 at the cell surface. Surprisingly, truncation of ALCAM severely impaired MMP-2 activation in a nude mouse xenograft model, in which we previously observed diminished primary tumor growth and enhanced melanoma metastasis. Comparative studies of two-dimensional monolayer and three-dimensional collagen-gel cultures revealed that extensive cell-to-cell contacts, wild-type ALCAM, and cell-to-matrix interactions were all indispensable for efficient conversion of pro–MMP-2 to its active form in metastatic melanoma cells. Truncated, dominant-negative ALCAM diminished MMP-2 activation via reduced transcript levels and decreased processing of MT1-MMP. Failure of the proteolytic cascade after selective ALCAM depletion by RNA interference was mainly due to incomplete MT1-MMP processing, which was otherwise promoted by extensive cell-to-cell contacts. These data attribute a novel signaling role to ALCAM in regulation of proteolysis and support its previously postulated sensor function in invasive growth. (Cancer Res 2005; 65(19): 8801-8)

Introduction

Matrix metalloproteinases (MMP) are Zn^{2+}-dependent proteinases whose expression is implicated in physiologic processes involving tissue remodeling and cell migration. MMP activity is also implicated in tissue invasion, metastasis, and angiogenesis (for reviews, see refs. 1–3). MMP-2/gelatinase A is the most abundant MMP and a marker of poor prognosis in various cancer types (4–6). MMPs are synthesized as preproenzymes and activated by proteolytic processing. According to current models, a complex of membrane type 1 MMP (MT1-MMP/MMP-14) and tissue inhibitor of metalloproteinases-2 (TIMP-2) recruits pro–MMP-2 from the extracellular milieu to the cell surface. Subsequent activation requires an additional molecule of active MT1-MMP and autocatalytic cleavage steps (7–11).

Interactions between extracellular matrix components and their cognate membrane receptors play an important role in the activation of the MT1-MMP/MMP-2 cascade in tumor cells. Clustering of integrin β1 chains or fibronectin binding promotes activation of MMP-2 (12, 13). Culturing metastatic melanoma cell lines in three-dimensional collagen lattices activates MT1-MMP and subsequently MMP-2 (14). Integrin αvβ3 binds MMP-2 and promotes its activation (15–18). The extracellular matrix may also determine the subcellular localization of MT1-MMP (19). Pro–MT1-MMP is activated intracellularly by the furin convertase (20) or on the cell surface by plasmin (21). Whereas the role of cell-to-matrix signaling in MMP-2 activation is relatively well documented, the contributions of other tissue architectural factors, such as homotypic cell-to-cell adhesion, are scarcely explored.

In human melanoma cell lines, expression of activated leukocyte cell adhesion molecule (ALCAM/CD166/MEMD) and MMP-2 activation correlate strongly with metastatic potential (22, 23). Furthermore, ALCAM expression was observed at the invasive front of vertical growth phase melanoma, indicating that ALCAM expression correlates with advanced melanoma tumor progression (24). Likewise, expression of MT1-MMP, TIMP-2, and activation of MMP-2 were also correlated to melanoma progression in xenografts and fresh human lesions (25).

ALCAM is a member of the immunoglobulin superfamily and has a characteristic $VVC_2C_2C_2$ domain structure. The NH_2-terminal V-type immunoglobulin folds constitute a homophilic ligand-binding module, whereas the membrane proximal C-type folds mediate oligomerization in *cis*. Together, these modules apparently contribute to the formation of a high-affinity, high-avidity molecular network that promotes cell-to-cell binding (26).

Interfering with endogenous ALCAM by expression of amino-terminally truncated ALCAM (ΔN-ALCAM) in metastatic melanoma cells increased cell migration *in vitro* and invasive growth in skin reconstructions, but reduced xenograft growth in nude mice while accelerating lung metastasis (27). These results suggested that proteolytic processing of ALCAM, as mimicked in these experiments by recombinant expression of ΔN-ALCAM, should induce a shift from primary tumor growth to invasive growth and metastasis. As these results also suggested that cell growth and invasion are separate phenotypic characteristics of tumor cells, we hypothesized that ALCAM expression coordinated these events in an oncogenic program that couples primary tumor growth and metastasis (for reviews, see refs. 28, 29).

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In view of the observed role of ALCAM as a rate-limiting factor in the transition from primary tumor growth to metastasis and as a potential substrate of metastasis-related proteinases (27), we addressed whether ALCAM-mediated cell adhesion would regulate MMP-2 activation. We show here that activation of MMP-2 depends on cell-to-cell adhesion and an intact ALCAM network. These results thus support a role for ALCAM in cell density–dependent activation of metastasis-promoting proteinase cascades.

Materials and Methods

Cell lines and reagents. The human melanoma cell lines BLM (metastatic; ALCAM-positive), BLM/ΔN-ALCAM (metastatic; ALCAM positive; truncated, dominant-negative ALCAM transfectant), 530 (nonmetastatic; ALCAM-negative), and 530/ALCAM (nonmetastatic; wild-type ALCAM transfectant; ref. 26), and the human fibrosarcoma cell line HT1080 (metastatic; ALCAM positive) were maintained in DMEM containing 10% FCS.

Antibodies. Mouse anti-ALCAM monoclonal antibody AZN-L50 (26) was used at 3 μg/mL for Western blotting. Rabbit anti–MT1-MMP polyclonal antibody AB815 (Chemicon, Hampshire, United Kingdom) was used at 0.5 μg/mL. Mouse anti-MMP2 monoclonal antibody Ab-3 (Merck Biosciences, Nottingham, United Kingdom) was used at 0.5 μg/mL for Western blotting. Mouse anti-TIMP2 monoclonal antibody Ab-1 (Merck) was used at 5 μg/mL for Western blotting. For immunohistochemical detection of ALCAM, monoclonal anti-ALCAM antibody NCL-CD166 (Novocastra, Newcastle, United Kingdom) was used at a dilution of 1:40. Goat anti-mouse immunoglobulin-coupled horseradish peroxidase (HRP) and swine anti-rabbit immunoglobulin-coupled HRP (DAKO, Glostrup, Denmark) were used as secondary antibodies in Western blotting at 1 μg/mL. Monoclonal antibodies 4B4 and Lia1/2 (Beckman Coulter, Fullerton, CA), both against the extracellular domain of human integrin β1, were used for coating of latex beads (see below).

Short interfering RNA–mediated activated leukocyte cell adhesion molecule knockdown. BLM cell cultures were transiently transfected using Oligofectamine reagent (Invitrogen, Carlsbad, CA) and a blunt-ended duplex of the RNA oligonucleotides 5′ -AAGCCCGAUGGCUCCCCAGUAUU-3′ and 5′-AAUACUGGGGAGCCAUCGGGCUU-3′ (RNA-TEC, Leuven, Belgium). Seventy-two hours posttranfection, the transfection procedure was repeated. Short interfering RNA (siRNA)–treated cells were detached from the culture vessel by a trypsin/EDTA treatment and used in subsequent experiments.

Coating of latex beads. A 10% (w/v) mixture of 3.0 μm latex beads (Sigma-Aldrich, Zwijndrecht, the Netherlands) was incubated with 100 μg/mL antibody solution in the presence of 50 mmol/L MES (pH 6.1). After overnight incubation on a rotator at 4°C, the beads were washed twice with PBS and blocked by 90 minutes incubation with 10 mg/mL bovine serum albumin (BSA) in PBS while rotating. The beads were washed twice in serum-free medium. Twenty microliters of bead suspension were added to cell cultures in 24-well plates. Conditioned media were isolated after 48 hours of incubation.

Three-dimensional collagen culture. Cells were cultured in either 5 mL (six-well plate, surface area 10 cm^2) or 1 mL (24-well plate, surface area 2 cm^2) cultures. Five-milliliter cultures were prepared by mixing 420 μL of 3.6 mg/mL rat tail collagen type I (BD Biosciences, Alphen a/d Rijn, the Netherlands) with 4 mL DMEM containing 10% serum and neutralized by 14 μL of 0.1 mol/L NaOH. Two hundred microliters of cells suspended in DMEM-containing serum were added after mixing and were incubated at 37°C. Gels formed after 10 to 15 minutes of incubation. After overnight incubation, the medium was removed, the gels were washed with PBS, and 2 mL serum-free DMEM were added. Conditioned medium was collected after 48 hours. For histochemical analyses, collagen gel cultures were supported by cell-free gels of half the volume of the gel that contained the cells to keep the specimens intact during processing.

Gelatin zymography. Conditioned media were mixed with 2× nonreducing sample buffer [150 mmol/L Tris-HCl (pH 7.0), 4% SDS, 22% glycerol] and incubated for 10 minutes at 65°C. Five microliters of media/sample buffer mixture, or tumor extract (prepared as described previously ref. 25), were separated on a 10% polyacrylamide gel containing 1 mg/mL gelatin (Merck). After electrophoresis, the gel was washed and incubated for 1 hour in 50 mmol/L Tris-HCl (pH 7.4) containing 2% Triton X-100 to renature the gelatinases. After washing twice in 50 mmol/L Tris-HCl (pH 7.4), the gel was incubated for 18 hours in 50 mmol/L Tris-HCl (pH 7.4), 5 mmol/L $CaCl_2$, 1% Triton X-100, 0.02% NaN_3, and subsequently stained by Coomassie brilliant blue R-250.

***In vivo* tumor growth.** Animal studies were approved by the Institutional Review Board for animal experiments. Male BALB/c athymic nude mice (*nu/nu*) were bred under specified pathogen-free conditions and used when 8 weeks old. Tumor cells were harvested using trypsin/EDTA, washed, and suspended in PBS. One hundred microliters of suspension containing 2×10^6 cells were inoculated s.c. Mice were inspected twice a week for general condition and local tumor growth was monitored. After 4 weeks, the animals were euthanized, tumors were dissected, frozen in liquid nitrogen, and stored at −70°C. Extracts from nonnecrotic tumor tissue were analyzed by gelatin zymography as described.

Histochemical staining of collagen gels. Collagen gels were fixed 4 hours in formaldehyde containing 0.2% Tween 20, dehydrated, and embedded in paraffin. Microtome specimens (6 μm) were stained by H&E staining procedure. For ALCAM visualization on paraffin-embedded material, an antigen retrieval procedure was used in which specimens were heated in a rice steamer in citrate buffer for 10 minutes. Specimens were incubated in normal horse serum, followed by anti-ALCAM monoclonal antibody, and subsequently by biotinylated secondary horse anti-mouse IgG (Vector Labs, Burlingame, CA). Bound antibody was visualized by incubation with avidin/biotin complex (Vector Labs), biotinylated HRP, and aminoethylcarbazole as the substrate.

Quantitative real-time PCR. mRNA was isolated using the RNeasy mini kit (Qiagen, Venlo, the Netherlands) and used as cDNA synthesis template using Superscript III reverse transcriptase (Invitrogen). For amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, the forward primer 5′-ATCTTCTTTTGCGTCGCCAG-3′ and reverse primer 5′-TTCCCCATGGTGTCTGAGC-3′ were used. For MT1-MMP cDNA, forward primer 5′-ATCATGGCACCCTTTTACCA-3′ and reverse primer 5′-CCTGACTCACCCCCATAAAG-3′ were used. For MMP-2 cDNA, forward primer 5′-GGCATTCAGGAGCTCTATGG-3′ and reverse primer 5′-GAGCGATGCCATCAAATACA-3′ were used. For TIMP-2 cDNA, forward primer 5′-GTAGTGATCAGGGCCAAAGC-3′ and reverse primer 5′-CAGGCCCTTTGAACATCTTT-3′ were used.

cDNA levels were determined using SYBR green fluorescence with the Abi-Prism 7000 (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Expression levels were evaluated as the number of cycle difference from GAPDH. Significant changes from control samples were determined using the standard Student's t test ($n = 3$). $P \geq 0.05$ was considered significant.

Western blotting. Collagen gels were centrifuged and washed with PBS. Cell extracts were prepared by using lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 140 mmol/L NaCl, 1 mmol/L $MgCl_2$, and 1 mmol/L $CaCl_2$] containing 1 mmol/L phenylmethylsulfonyl fluoride and proteinase inhibitor cocktail. Protein concentrations were determined using the BCA kit (Pierce, Rockford, IL). Twenty micrograms of cell lysate were separated by SDS-PAGE on 10% PAA gels for MT1-MMP and MMP-2 detection or 15% PAA gels for TIMP-2 detection, and transferred to polyvinylidene difluoride membrane. Bound primary antibodies were visualized by appropriate HRP-coupled secondary antibodies and enhanced chemiluminescence using the ECL Plus Western blotting Detection System (Amersham Biosciences, Roosendaal, the Netherlands).

Results

Influence of cell-to-cell interactions on MMP-2 activation induced by integrin clustering in monolayer culture. To evaluate the relative contributions of cell-to-cell and cell-to-matrix interactions to MMP-2 activation in metastatic melanoma BLM cells, we used a well-defined, two-dimensional monolayer culture system in which cell-to-matrix interactions were mimicked by overlaying cells with anti-integrin β1 antibody-coated beads. In DOV13 ovarian carcinoma cells, this approach induced integrin

clustering and activation of MMP-2 (13). Nonconfluent (5×10^3 cm^{-2}) and 100% confluent (2.5×10^4 cm^{-2}) cell monolayers were overlaid with latex beads coated with the anti-integrin β1 antibody 4B4 or with BSA as a control. Conditioned media from these cultures were analyzed by gelatin zymography. Nontreated BLM cultures and those treated with BSA-coated beads did not show conversion of pro–MMP-2 (72 kDa) to active MMP-2 (59 kDa; Fig. 1*A*, *top*). Confluent BLM cultures treated with 4B4-coated beads showed slight but reproducible conversion to intermediate (62 kDa) and active (59 kDa) MMP-2, whereas in nonconfluent cultures no significant activation was observed. Similar results were obtained with the anti-integrin β1 antibody Lia1/2 (data not shown). Therefore, MMP-2 activation in response to clustering of integrin β1 clearly depended on cell confluence.

To assess the role of cell-to-cell interactions more specifically, we tested how disruption of the ALCAM network in BLM cells affected MMP-2 activation. The BLM/ΔN-ALCAM cell line expresses an ALCAM deletion mutant as well as wild-type ALCAM at a similar level as the parental BLM cells (26). Interestingly, 4B4-coated beads failed to induce MMP-2 activation in BLM/ΔN-ALCAM cells, regardless of culture confluence (Fig. 1*A*, *bottom*). Alternatively, we used synthetic siRNAs and selectively knocked down ALCAM protein to levels virtually undetectable by Western blotting (Fig. 1*B*). In overconfluent (5×10^4 cm^{-2}) siRNA-transfected cultures, addition of anti-integrin β1-coated beads induced only slight pro–MMP-2 cleavage, whereas a stronger, dose-dependent increase in the cleavage of pro–MMP-2 was observed in mock-transfected control cultures (Fig. 1*C*).

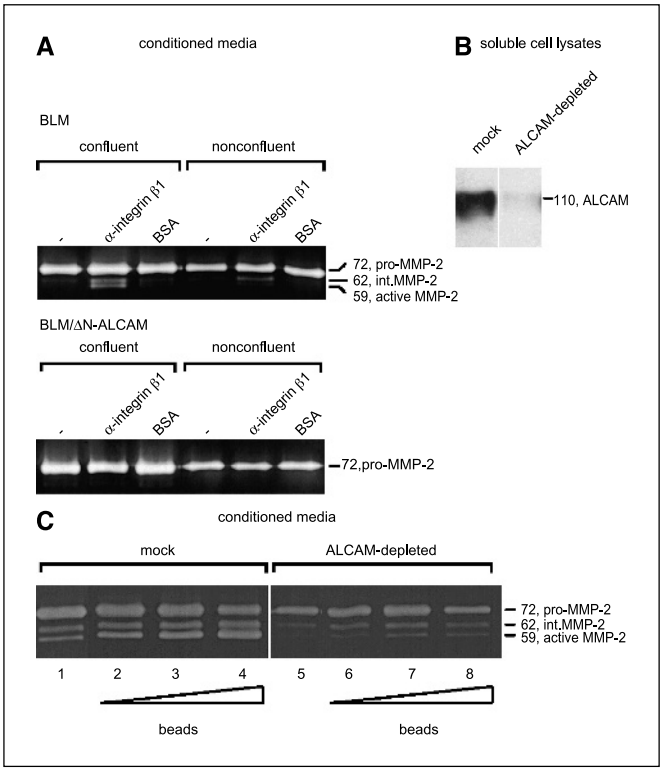

Figure 1. Integrin β1 clustering stimulates MMP-2 activation in confluent monolayers only in the presence of an intact ALCAM network. *A,* confluent (2.5×10^4 cm^{-2}) and nonconfluent (5×10^3 cm^{-2}) BLM or BLM/ΔN-ALCAM cells were cultured in monolayers and treated with beads coated with anti-integrin β1 antibody 4B4, BSA as control, or were left untreated. The samples of the nonconfluent cultures were loaded in 5-fold volume for normalization of cell numbers. *B,* cell extracts of mock-transfected and anti-ALCAM siRNA-treated BLM cells were probed with anti-ALCAM antibody AZN-L50. *C,* 5×10^4 cm^{-2} mock-transfected or ALCAM-depleted BLM cells were cultured as in (*A*). Cells were treated with 5 μL (*lanes 2* and *6*), 10 μL (*lanes 3* and *7*), or 20 μL (*lanes 4* and *8*) of bead suspension coated with antibody 4B4. Untreated mock-transfected (*lane 1*) and ALCAM-depleted BLM cells (*lane 5*) served as controls. Conditioned media samples were analyzed by gelatin zymography. Positions of pro–, intermediate, and active MMP-2 with sizes in kDa are indicated.

These results showed that in monolayer BLM cultures, MMP-2 activation promoted by antibody-induced clustering of integrin β1 depended on the degree of confluence (Fig. 1*A*), ALCAM protein levels (Fig. 1*B* and *C*), and an intact ALCAM network (Fig. 1*A*). Hence, cell-to-cell interactions involving ALCAM were required for MMP-2 activation as well as cell-to-matrix interactions mediated by integrin β1.

Influence of increasing cell density and an intact activated leukocyte cell adhesion molecule cell-to-cell adhesion network on activation of MMP-2 in three-dimensional collagen gel cultures. We next studied the requirements for MMP-2 activation in a more complex, three-dimensional culture model. Culturing metastatic melanoma cells in type I collagen gels provided improved conditions for MMP-2 activation (14). Embedding cells in these gels exposed integrin β1 to a common ligand in a structured lattice resembling stroma, but influences of cell-to-cell adhesion were not studied in this model. If integrin clustering were the major inducer, one could expect significant activation of MMP-2 to occur regardless of cell density in collagen gel.

Increasing numbers of BLM cells, BLM/ΔN-ALCAM cells, nonmetastatic melanoma 530 cells (ALCAM-negative), and 530 cells stably expressing ALCAM (530/ALCAM) were grown in collagen gels and as monolayers in 24-well plates. Conditioned media from 530 and 530/ALCAM cells grown in collagen gels showed a 72 kDa band corresponding to pro–MMP-2 in zymograms, whereas media from monolayer cultures of the same cell lines did not (Fig. 2*A*, *two top panels*). Neither of the two cell lines showed MMP-2 activation in collagen regardless of cell density and ALCAM expression. A fainter 92 kDa band, corresponding to pro–MMP-9/gelatinase B, was also visible in both cell lines when grown in collagen gels, but not in monolayers.

BLM cells showed stronger expression of pro–MMP-2 in either collagen or monolayer culture than 530 or 530/ALCAM and also expressed pro–MMP-9 in collagen culture (Fig. 2*A*, *third panel*). In monolayers in general and in low-density BLM collagen cultures (10^3 cells per gel), active MMP-2 (59 kDa band) was not or barely detectable, while MMP-2 activation was very efficient in collagen gels at high cell density (10^5 cells per gel). For a normalized comparison, 10- and 100-fold dilutions, respectively, of conditioned media from the intermediate (10^4) and high (10^5) cell density collagen gel cultures showed stronger MMP-2 activation than the undiluted 10^3 culture, underscoring that MMP-2 activation was enhanced with increasing cell density (not shown).

BLM/ΔN-ALCAM cells cultured in collagen gel showed no activation of MMP-2 at all at the low and intermediate cell densities. Only at the highest density, faint intermediate and activated MMP-2 forms were visible (Fig. 2*A*, *bottom panel*). Hence, the apparent disruption of an intact ALCAM network inhibited MMP-2 activation in BLM cells in collagen gel.

To investigate whether the dependence of MMP-2 activation on cell density was a more general phenomenon, human fibrosarcoma HT1080 cells were seeded in collagen gels and monolayers in varying densities in six-well plates (note that cell numbers were 10-fold higher to compensate for the larger surface area and volume). This cell line expresses ALCAM on the mRNA and the

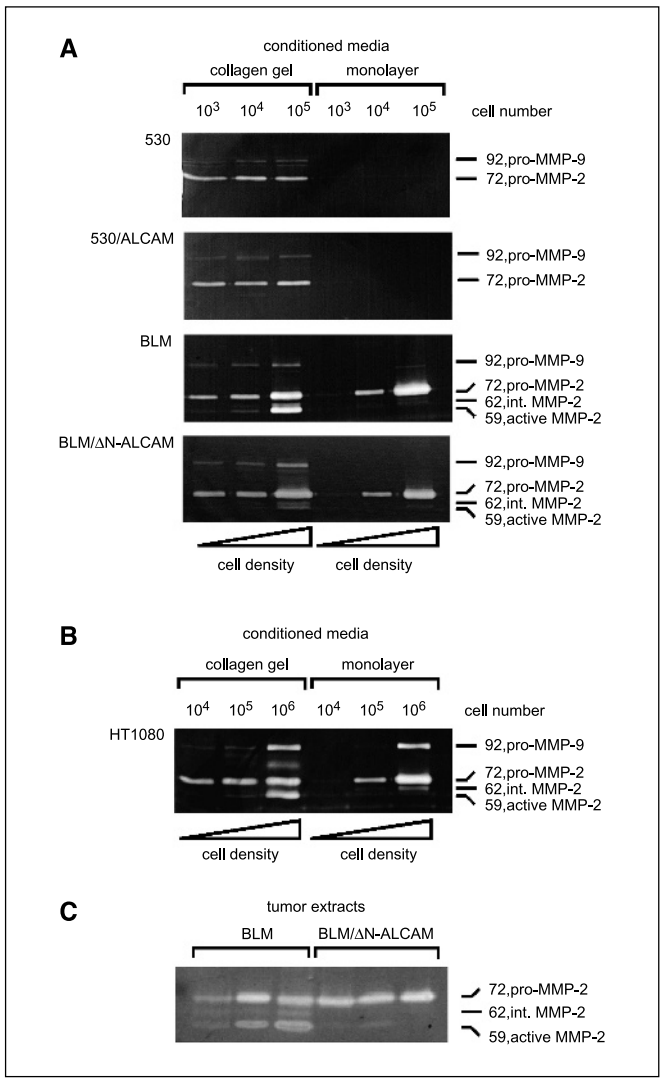

Figure 2. Cleavage of pro–MMP-2 to active MMP-2 in metastatic cells is promoted in collagen gels, requires high cell numbers, and depends on formation of an intact ALCAM network at cell-to-cell junctions. *A,* 530, 530/ALCAM, BLM, and BLM/ΔN-ALCAM melanoma cell lines were cultured in collagen gels or as monolayers in 24-well plates. Conditioned media were analyzed by gelatin zymography. *B,* HT1080 cells were cultured in collagen lattices and monolayers (six-well plates). Conditioned media were analyzed by gelatin zymography. After overnight incubation, contraction of collagen gels was evident in high-density BLM and HT1080 cultures, reduced in BLM/ΔN-ALCAM cells, and absent in 530 and 530/ALCAM cells. *C,* BLM and BLM/ΔN-ALCAM cells were s.c. injected into nude mice and extracts of dissected tumors were analyzed by gelatin zymography. Results of three independent tumors per cell line are shown. Positions of pro–MMP-9, pro-, intermediate, and active MMP-2 with sizes in kDa are indicated.

protein level (ref. 22 and data not shown). As observed in BLM melanoma cells, significant pro–MMP-2 conversion in collagen culture occurred only at the highest cell density (Fig. 2*B*). In HT1080 monolayers, very little conversion of pro–MMP-2 occurred and only a faint intermediate band was visible at the highest cell density. Similar results were obtained in MV3 metastatic melanoma cells (data not shown). This indicated that cell density–dependent activation of MMP-2 in collagen lattices also occurred in other tumor cell lines with endogenous ALCAM expression and was not restricted to melanoma. Strikingly, whereas cell-to-matrix interactions and clustering of integrin β1 were limiting conditions in confluent monolayer cell culture (Fig. 1), cell-to-cell interactions seemed to be limiting factors for MMP-2 activation in a three-dimensional collagen gel (Fig. 2).

Effects of activated leukocyte cell adhesion molecule truncation on MMP-2 activation in nude mouse xenografts. To confirm that disruption of the ALCAM network also inhibited MMP-2 activation *in vivo*, BLM and BLM/ΔN-ALCAM cells were s.c. injected into nude mice. After 4 weeks of incubation, tumors were collected. Extracts from healthy, nonnecrotic tissue were analyzed by gelatin zymography (Fig. 2*C*). BLM tumors showed extensive activation of MMP-2, whereas BLM/ΔN-ALCAM tumors did not, indicating that interference with ALCAM network formation also inhibits MMP-2 activation in mouse xenograft tumors.

Correlation between pro–MMP-2 activation and cell-to-cell contact formation and the need of activated leukocyte cell adhesion molecule. The observation that collagen gel–induced MMP-2 activation occurred only at higher cell densities and was dependent on ALCAM function suggested that cell-to-cell contacts were necessary for MMP-2 activation in addition to clustering of integrin β1. To determine if MMP-2 activation actually coincided with cell-to-cell contact formation, BLM and BLM/ΔN-ALCAM cells were seeded in varying cell densities in collagen gels. Six-well plates were used to facilitate histologic processing. After incubation and collection of conditioned media, the collagen cultures were processed for paraffin embedding and histochemistry. As in previous experiments, gelatin zymograms of the conditioned media showed an increase in MMP-2 activation as BLM cell density increased from 10^4 to 10^6 cells per gel, and virtually no activation was observed in BLM/ΔN-ALCAM cells seeded at the same densities (Fig. 3*A*). H&E-stained collagen gel sections of both BLM and BLM/ΔN-ALCAM cells seeded at low cell density showed few cell-to-cell contacts, whereas at the highest cell density extensive cell-to-cell contacts were visible (Fig. 3*B*). Despite the progressive formation of cell-to-cell contacts (Fig. 3*B, right column*), BLM/ΔN-ALCAM cells were unable to activate MMP-2 (Fig. 3*A*). Thus, MMP-2 activation and cell-to-cell contact formation did coincide in BLM cells, but not in BLM/ΔN-ALCAM cells.

Anti-ALCAM siRNA-treated and mock-transfected BLM cells were cultured in collagen gels at high cell density (10^6 cells in 24-well plates). Zymograms showed that mock-transfected control BLM cells cleaved pro–MMP-2 efficiently, whereas MMP-2 activation in siRNA-treated BLM cells was much less efficient, indicating that ALCAM was necessary for collagen-induced MMP-2 activation in BLM cells (Fig. 3*C*). The gels were processed for histochemistry as before. Immunohistochemical staining of specimens with anti-ALCAM antibody showed clear ALCAM signals in mock-transfected cells, whereas siRNA-treated BLM cells only showed background signals (Fig. 3*D, top row*). Mock-transfected BLM cells showed extensive cell clustering in contrast to siRNA-treated cells (Fig. 3*D, bottom row*). Taken together, these results show that ALCAM-mediated cell-to-cell adhesion favors cell clustering and efficient MMP-2 activation in collagen gel.

Effects of activated leukocyte cell adhesion molecule status on MT1-MMP, TIMP-2, and MMP-2 messenger RNA expression. Current models state that assembly of a ternary complex of MT1-MMP, TIMP-2, and pro–MMP-2 is involved in the MMP-2 activation cascade. We, therefore, investigated whether any changes occurred in expression of these components upon disruption of the ALCAM network or depletion of ALCAM. Messenger RNA levels of MT1-MMP, TIMP-2, and MMP-2 were determined by quantitative real-time PCR in BLM, BLM/ΔN-ALCAM, and in BLM cells transfected with anti-ALCAM siRNA in monolayer culture. The only significant

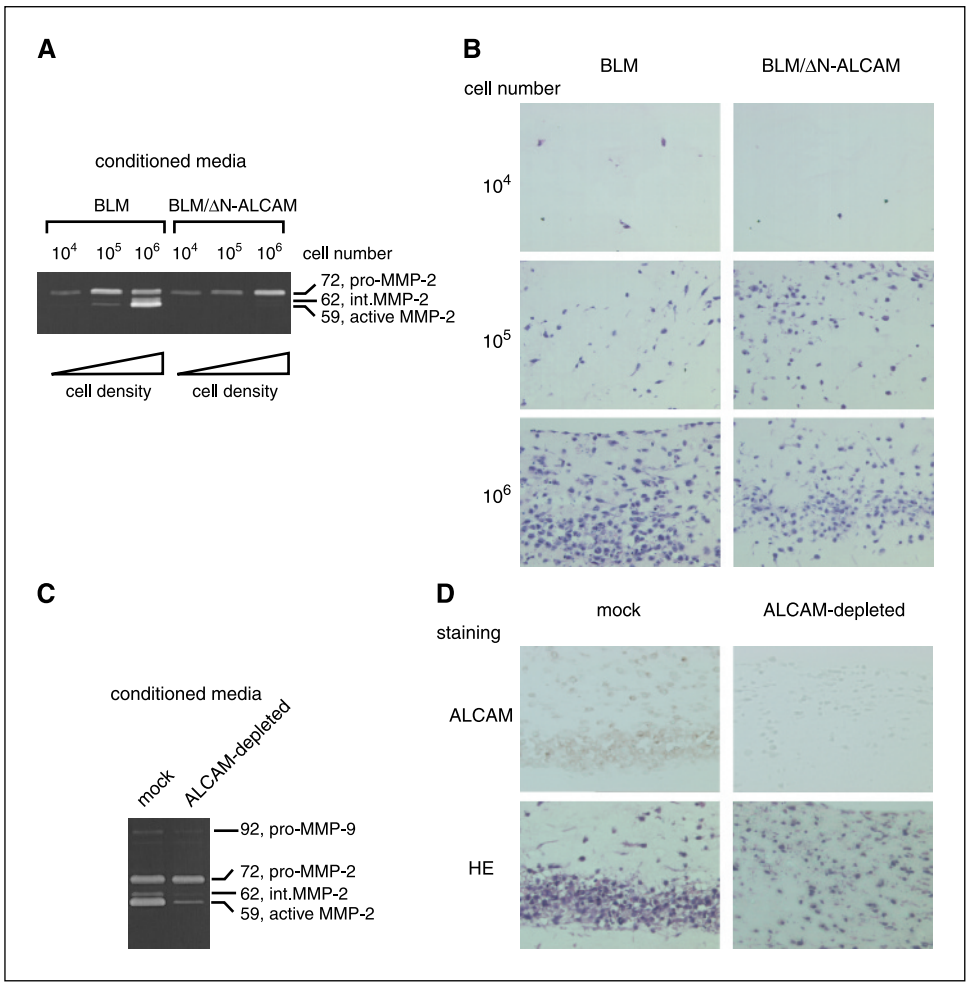

Figure 3. Activation of MMP-2 coincides with cell-to-cell contact formation in BLM cells but not in BLM/ΔN-ALCAM cells and is inhibited by ALCAM depletion. *A* and *B,* BLM and BLM/ΔN-ALCAM cells were cultured in varying cell numbers in collagen gels (six-well plates). *A,* gelatin zymogram of the conditioned media from cultures shown in (*B*). Positions of pro–, intermediate, and active MMP-2 with sizes in kDa are indicated. *B,* H&E-stained sections of formalin-fixed, paraffin-embedded collagen cultures of BLM cells (*left column*) and BLM/ΔN-ALCAM cells (*right column*) at increasing cell densities from 10^4 (*top row*), to 10^5 (*middle row*), to 10^6 (*bottom row*) cells per gel. *Bar,* 100 μm. *C* and *D,* 10^6 mock-transfected and ALCAM-depleted BLM cells were cultured in collagen gels in 24-well plates and processed as in (*A*). *C,* gelatin zymogram of the conditioned media from cultures shown in (*D*). Positions of pro–MMP-9, pro-, intermediate, and active MMP-2 with sizes in kDa are indicated. *D,* immunohistochemical stainings using anti-ALCAM antibody (*top row*) and H&E stainings (*HE*, *bottom row*) of mock-transfected control BLM cells (*left column*) and ALCAM-depleted BLM cells (*right column*). *Bar,* 100 μm.

change observed concerned the reduced MT1-MMP expression levels in BLM/ΔN-ALCAM cells, whereas MMP-2 and TIMP-2 levels were not significantly affected (Fig. 4*A*). Anti-ALCAM siRNA treatment did not affect MT1-MMP and MMP-2 expression, whereas TIMP-2 expression was slightly lowered upon siRNA treatment (Fig. 4*B*). The reduced expression of MT1-MMP may explain the decreased MMP-2 activation in BLM/ΔN-ALCAM cells. On the other hand, the reduction in MMP-2 activation in ALCAM-depleted cells is likely due to other causes as no changes in mRNA expression levels were observed in siRNA-treated cells.

Regulation of MT1-MMP expression and activation by activated leukocyte cell adhesion molecule network formation. Whereas the previous zymograms (Figs. 1–3) showed MMP-2 isoforms secreted from cells into the growth medium, we wanted to study expression of cell-associated proteins involved in MMP-2 activation in the next series of experiments. With this purpose, we prepared soluble cell lysates of BLM and BLM/ΔN-ALCAM cells grown at high cell densities in collagen gels or monolayers and analyzed them by Western blotting. MT1-MMP in BLM cells grown in collagen gels was visible as a 65 kDa precursor band and as an active 60 kDa band (Fig. 5*A*; ref. 30). The same bands were present in BLM cells grown in monolayer, but the prominent appearance of an additional 64 kDa band and the relatively lower intensity of the 60 kDa band indicated that processing of MT1-MMP was incomplete in monolayers. Consistent with the reduced mRNA levels (Fig. 4*A*), expression of MT1-MMP protein in BLM/ΔN-ALCAM cells was lower in general, and the major band in collagen was the 65 kDa band. In the monolayers, the most prominent MT1-MMP species was the partially processed 64 kDa band. Overexpression of ΔN-ALCAM apparently reduced both expression levels of MT1-MMP and its processing.

The MMP-2 antibody recognized 72, 62, and 59 kDa bands in BLM cells in collagen, and only the upper two bands were observed in monolayers (Fig. 5*B*). In BLM/ΔN-ALCAM cells in collagen, the most prominent band was the 72 kDa band, indicating a lack of pro–MMP-2 processing. A faint 59 kDa band was also visible, but the intensity of this band was clearly reduced compared with BLM cells. The prominent presence of the 62 kDa band in monolayers (BLM/ΔN-ALCAM and BLM) pointed to incomplete processing. These results were in agreement with the previous zymograms (Figs. 2 and 3), showing a general lack of active MMP-2 in the conditioned media of BLM/ΔN-ALCAM cultures. The expression levels of TIMP-2 in BLM and BLM/ΔN-ALCAM cultures were similar (Fig. 5*C*). In summary, these results indicate that the protein levels of MT1-MMP are generally decreased in BLM/ΔN-ALCAM cells. Furthermore, MT1-MMP processing to the active 60 kDa form is less efficient in monolayer culture and upon overexpression of ΔN-ALCAM also in collagen gel. The reduced conversion of pro–MMP-2 to its active form may thus be attributed to diminished levels of the 60 kDa isoform of MT1-MMP.

Because anti-ALCAM siRNA treatment offered an alternative strategy to impair the MT1-MMP/MMP-2 activation cascade, soluble

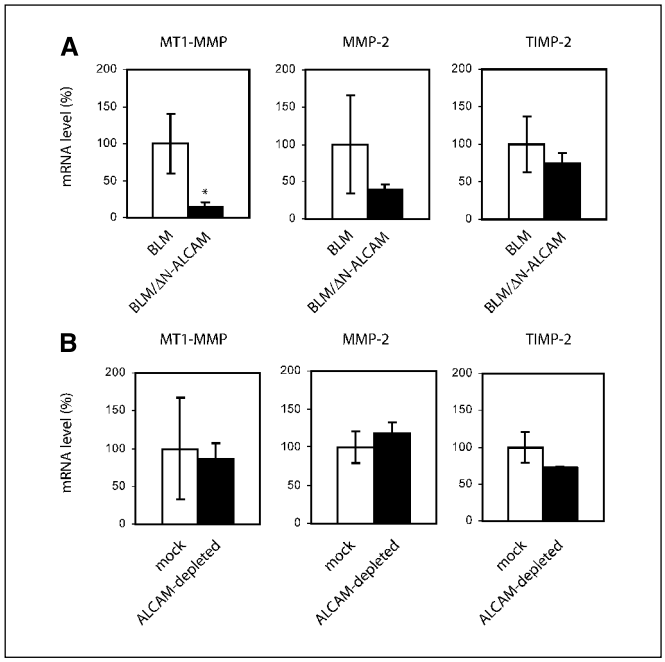

Figure 4. Quantitative real-time PCR analysis of *MT1-MMP*, *MMP-2*, and *TIMP-2* gene expression. *A,* analysis of BLM cells at a density of 5×10^5 cm^{-2} (*white columns*) and BLM/ΔN-ALCAM cells at a density of 4.5×10^5 cm^{-2} (*black columns*). *B,* analysis of mock-transfected (*white columns*) and anti-ALCAM siRNA-treated BLM cells (*black columns*) at a density of 2×10^5 cm^{-2}. The expression level of each gene was set at 100% for BLM cells in (*A*), and for mock-transfected BLM cells in (*B*). *Columns,* means (n = 3); *bars,* SD. *Significant difference ($P \leq 0.05$, Student's t test).

cell lysates of the transfected cells grown in collagen gels were analyzed by Western blotting. Whereas mock-transfected BLM cells showed 65 and 60 kDa bands in an MT1-MMP Western blot, only 65 and 64 kDa bands were visible in ALCAM-depleted cells (Fig. 6*A*). This showed that, although anti-ALCAM siRNA treatment does not affect MT1-MMP expression (compare with Fig. 4*B*), ALCAM is necessary for the processing of MT1-MMP to its active 60 kDa form. The incomplete processing of MT1-MMP in ALCAM-depleted cells was accompanied by poor activation of MMP-2 (Fig. 6*B*; compare with zymography results in Fig. 3*C*). TIMP-2 levels were unaltered (Fig. 6*C*). Together, these results show that ALCAM is involved in expression and processing of MT1-MMP and, thus, affects the subsequent conversion of pro–MMP-2 to active MMP-2.

Discussion

Tissue homeostasis and extracellular matrix remodeling rely heavily on control of proteolytic events. Invasive tumor growth is commonly accompanied by MMP activation and proteolysis of matrix proteins. In particular, activation of the MT1-MMP/MMP-2 cascade is linked to melanoma progression (25, 31). Although the (patho)physiologic conditions for activation of these proteinases are not completely understood, it is known that integrin receptor clustering contributes to up-regulation of pericellular MMP-2 activity (13, 32). In this study, we show that cell-to-cell contacts are at least as important as cell-to-matrix interactions in this process. This is remarkable because it implies that cell proliferation in a developing tissue may, by itself, generate signals to activate proteolytic cascades.

When cultured in collagen gels, BLM cells up-regulate expression of the collagen receptor, integrin $\alpha 2\beta 1$ (33), and readily activate MMP-2 (14). The contribution of cell-to-cell adhesion to MMP-2 activation in this model has not been studied thus far. The dependence of the MT1-MMP/MMP-2 cascade on cell-to-cell adhesion was clearly shown by the observations that MMP-2 activation induced by treatment with anti-integrin β1-coated beads (Fig. 1) or by culture in collagen gels (Figs. 2 and 3) occurred only in dense BLM cell cultures, but not in sparse cultures.

The functional status of the cell adhesion molecule ALCAM/CD166 was a primary determinant for the regulation of MMP-2 activation in the melanoma cells analyzed because inhibition of the MMP-2 cascade was achieved both by overexpression of a truncated ALCAM mutant, which attenuated cell-to-cell adhesion, or by anti-ALCAM siRNA treatment. Surprisingly, the present analysis also revealed that impaired MMP-2 activation in nude mice xenografts accompanied the enhanced invasiveness of metastatic melanoma cells after overexpression of NH_2-terminally truncated ALCAM (Fig. 2*C*; ref. 27).

As ALCAM functions as a homophilic cell adhesion molecule in BLM melanoma cells, our data suggest that ALCAM-mediated cell-to-cell adhesion contributes to MMP-2 activation. Indeed, we have found a strong correlation between pro–MMP-2 cleavage and the incidence of cell-to-cell contacts in BLM cells, but not in BLM/ΔN-ALCAM cells (Fig. 3*A* and *B*). Together, these experiments clearly indicate that activation of MMP-2 in metastatic melanoma cells requires (*a*) cell-to-cell adhesion, (*b*) wild-type ALCAM, and (*c*) integrin clustering.

The effects of functional interference with ALCAM could be attributed to diminished mRNA expression and/or incomplete

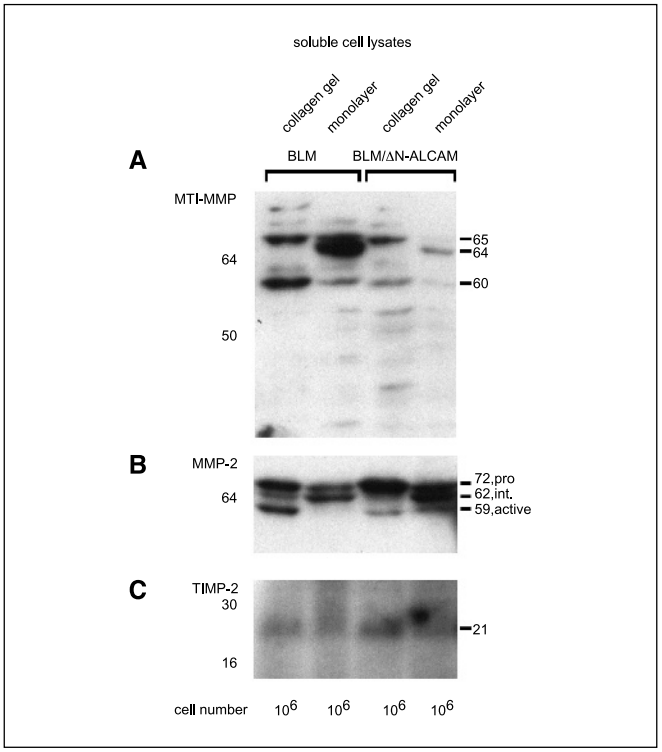

Figure 5. Generation of active MT1-MMP is promoted in collagen gels, depends on an intact ALCAM network, and supports conversion of pro–MMP-2 to active MMP-2. One million BLM and BLM/ΔN-ALCAM cells were grown in collagen gels or as monolayers in six-well plates for 48 hours. Cell lysates were analyzed by Western blotting using polyclonal antiserum against MT1-MMP (*A*), monoclonal antibodies against MMP-2 (*B*), and monoclonal antibodies against TIMP-2 (*C*). Sizes (in kDa) of molecular weight markers (*left*) and positions of protein bands (*right*) are indicated.

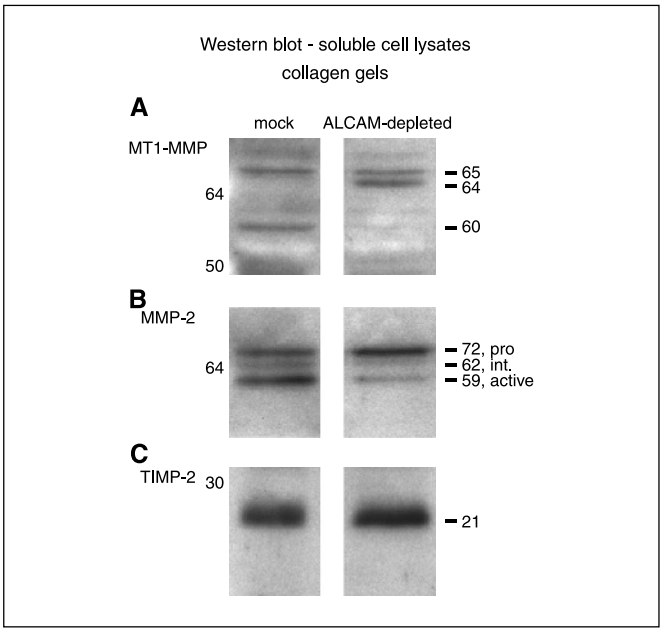

Figure 6. MT1-MMP activation depends on ALCAM expression. One million mock-transfected and anti-ALCAM siRNA-treated BLM cells were grown in collagen gels in six-well plates for 48 hours. Cell extracts were analyzed by Western blotting using polyclonal antiserum against MT1-MMP (*A*), monoclonal antibodies against MMP-2 (*B*), and monoclonal antibodies against TIMP-2 (*C*). Sizes (in kDa) of molecular weight markers (*left*) and positions of protein bands (*right*) are indicated.

processing of MT1-MMP (Figs. 4-6). BLM cells efficiently promoted MT1-MMP processing upon culture in collagen gels, whereas only poor processing occurred in monolayers (in agreement with ref. 14). ΔN-ALCAM expression lowered MT1-MMP expression and processing. The observed lack of MMP-2 activation after ALCAM depletion by siRNA could not be attributed to any changes in mRNA or protein levels of MT1-MMP and merely affected MT1-MMP processing. These observations indicate that coordinate and progressive *cis* and *trans* clustering of ALCAM should generate a signal and stimulate productive MT1-MMP processing and subsequent MMP-2 activation.

Cell adhesion and matrix metalloproteinase activity. The observation that density-dependent MMP-2 activation occurs in ALCAM-positive human fibrosarcoma HT1080 cells suggests that this phenomenon is not restricted to melanoma. Besides, other cell adhesion molecules may perform similar roles as ALCAM. The extracellular MMP inducer EMMPRIN/CD147 is highly expressed in tumor cells and induces adjacent fibroblasts to express MMPs (34–37). Furthermore, E-cadherin may control MT1-MMP expression levels through the Wnt signaling pathway, but it is likely that these effects are cell type dependent (38–41).

MMPs were traditionally thought to promote metastasis by breakdown of extracellular matrix proteins, thus allowing tumor cells to pass through tissue barriers. This view was challenged because various MMP inhibitors proved unsuccessful at inhibiting the metastatic process in clinical trials (42). Also, it was shown that invasive tumor cells could compensate for blocked proteolysis by changing their migratory strategies (43). BLM/ΔN-ALCAM cells showed increased invasive growth in skin reconstructions and increased metastasis with reduced primary xenograft growth in nude mouse models (27). Despite the apparent inhibition of MMP-2 activation (this report), BLM/ΔN-ALCAM cells display increased invasive behavior, suggesting that these properties should be less dependent on MMP-2 activity.

Our data indicate a novel role for ALCAM in the cell density–dependent regulation of MT1-MMP expression and processing and subsequent activation of MMP-2. Thus, in metastatic melanoma BLM cells, MMP-2 activation is regulated by cell-to-cell interactions, mediated by ALCAM, in addition to cell-to-matrix interactions involving integrin β1. The importance of ALCAM in this mechanism suggests that ALCAM acts as a cell density sensor and initiates a signal toward MMP-2 activation. We hypothesize that the MMP-2 activating stimulus in three-dimensional collagen gels originates from extensive ALCAM-ALCAM clustering interactions in cell-to-cell junctions. Thus, an effective stimulus should exceed a minimum threshold as a function of increasing cell number and density. It is noteworthy that in invasive lesions, tumor cells display polarization with regard to cell-to-cell and cell-to-matrix binding. At the invasive front in particular, tumor cells engage in both cell-to-matrix and cell-to-cell binding. It has been shown that ALCAM expression and MMP-2 activation occur at this site in melanocytic tumors (24, 25). Together with our observations that ALCAM regulates cell density–dependent expression of tumor progression–related genes,[5] these results imply that ALCAM contributes positively to melanoma metastasis, and, therefore, explain why expression of a cell adhesion molecule that mediates homotypic cell adhesion correlates with metastatic potential. Furthermore, the regulation of proteolytic activity by progressive cell clustering suggests a novel aspect of growth control in transformed cells. Thus, invasive growth and metastasis may be elements of an intrinsic response to local cell saturation in cells unable to obey growth arrest signals, offering an alternative mode to react to crowding conditions in the tumor microenvironment.

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Activated Leukocyte Cell Adhesion Molecule (ALCAM/CD166/MEMD), a Novel Actor in Invasive Growth, Controls Matrix Metalloproteinase Activity

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