ALCAM, Activated Leukocyte Cell Adhesion Molecule, Influences the Aggressive Nature of Breast Cancer Cells, a Potential Connection to Bone Metastasis

SIMON DAVIES and WEN G. JIANG

Metastasis and Angiogenesis Research Group, Cardiff University School of Medicine Heath Park, Cardiff CF14 4XN, United Kingdom

Abstract. Introduction: ALCAM, activated leukocyte cell adhesion molecule, is connected to the progression of certain solid tumours and has been shown to be a prominent feature for tumours that subsequently developed bone metastasis. The present study investigated the biological influence of ALCAM on breast cancer cells in connection with bone biological environment. Materials and Methods: Suitable breast cancer cells were transfected with either the ALCAM expression construct or anti-ALCAM transgene, to create sublines that had differential expression of ALCAM. The growth, migration and invasion of the cells were evaluated in the presence or absence of matrix proteins prepared from human bones. Results: ZR-751ΔALCAM (ALCAM knockdown) and MDA-MB-231ALCAMexp (overexpressing ALCAM) were constructed. MDA MB-231ALCAMexp cells showed a slower rate of growth compared with control cells. However, in the presence of bone matrix proteins, MDA MB-231ALCAMexp showed a significantly reduced rate of growth, p<0.01 vs. control cells. In contrast, ZR-751ΔALCAM cells grew faster compared with control cells. MDA MB-231ALCAMexp displayed a significantly reduced (p=0.012) and ZR-751ΔALCAM cells significantly increased invasiveness (p=0.02) vs. their respective controls cells. In an ECIS-based cell migration assay, MDA-MB-231ALCAMexp cells showed marked reduction in migration. Inclusion of bone matrix proteins therefore further reduced the migration speed of MDA MB-231ALCAMexp cells. Conclusion: Loss of ALCAM in breast cancer cells facilitates the invasive behaviour of breast cancer and high levels of ALCAM in the cells have a suppressive role in the aggressive nature of breast cancer cells.

Correspondence to: Wen G. Jiang, Metastasis and Angiogenesis Research Group, Cardiff University School of Medicine, Heath Park, Cardiff CF14 4XN, U.K. Tel: +44 2920742895, Fax: +44 2920761623, e-mail: jiangw@cf.ac.uk

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Activated leukocyte cell adhesion molecule (ALCAM), also known as melanoma metastasis clone D (MEMD) or CD166, was first identified on activated leukocytes and in haemopoietic stem cells and myeloid progenitors (1, 2). The gene encoding ALCAM is located on the long arm of human chromosome 3 (3q13.1-q13.2) (3). ALCAM is a glycoprotein of the immunoglobulin superfamily that is involved in homophilic adhesion and in heterotypic binding to the lymphocyte cell surface receptor CD6 (4, 5). ALCAM- mediated cell adhesion is mediated by both homophilic (ALCAM-ALCAM) and heterophilic (ALCAM-CD6) (6) interactions between cells, with the heterophilic interactions being approximately 100 times stronger than the homophilic interactions (7). The influence of ALCAM on cell adhesion has inspired a number of studies to evaluate its expression in human tumours including melanoma, prostate cancer, breast cancer, colorectal carcinoma, bladder cancer and oesophageal squamous cell carcinoma. ALCAM expression has been shown to have clinical implications. In malignant melanoma, approximately half of the cases investigated were found to have ALCAM positivity and the expression is seen in the vertical growth phase of melanoma (6). In prostate cancer, colorectal carcinoma and oesophageal squamous cell carcinoma, ALCAM expression was lost in high-grade tumours (8-10). ALCAM has also been shown to be involved in capillary tubule formation (11) and in vessel invasion into cartilage in vitro (12). Degen et al. were the first to look for the presence of ALCAM in breast cancer, and performed northern blot analysis of MEMD (a partner to ALCAM) mRNA and found expression in the MCF-7 mammary carcinoma cell line. Studies have more recently shown ALCAM in resected breast tumours (13) and King et al. shown a decreased level of ALCAM expression correlating with the nodal involvement, the grade, the TMN stage, the Nottingham Prognostic Index NPI and the clinical outcome (local recurrence and death) (13). These finding have recently been substantiated by Jezierska et al., who similarly concluded that low ALCAM concentrations correlated with an aggressive phenotype (14).
The published data on the function of ALCAM in breast cancer are not wholly consistent, there is however growing evidence in support of initial findings showing that a reduced level of ALCAM expression is an indicator of poor prognosis in breast cancer (13, 14). It has been reported recently that ALCAM expression is particularly aberrant in breast tumours which subsequently develop bone metastasis, and that ALCAM expression level is linked to both the clinical outcome and the presence of bone metastasis (15).

It is not clear how ALCAM expression may affect the cellular behaviour of breast cancer cells, and whether the expression profile may influence osteotrophy of breast cancer cells. In the present study, a series of breast cancer sublines with different expression of ALCAM were developed and their biological behaviour was tested, including growth, migration and invasiveness. Furthermore, these parameters were tested in the presence of bone matrix proteins.

**Materials and Methods**

*Materials.* Human breast cancer cell lines, MDA MB-231 and ZR 7-51, were obtained from ATCC (American Type Culture Collection, Maryland, USA). Other cells were obtained from ECACC (European Collection of Animal Cell Culture, Salisbury, England, UK). The endothelial cell line HECV was from Interlab Cell line Collection (ICLC, Naples, Italy). Recombinant human hepatocyte growth factor/scatter factor (rhHGF/SF) was a gift from Dr. T. Nakamura, Osaka University Medical School, Osaka, Japan. Matrigel (reconstituted basement membrane) was obtained from Collaborative Research Products (Bedford, MA, USA). Transwell plates equipped with a porous insert (pore size 8 μm) were obtained from Becton Dickinson Labware (Oxford, UK). DNA gel extraction and plasmid extraction kits were obtained from Sigma (Poole, Dorset, UK). Anti-actin and anti-GAPDH were from Santa Cruz Biotechnologies Inc., (Santa Cruz, CA, USA). Molecular biology grade agarose and DNA ladder were obtained from Invitrogen (Carlsbad, CA, USA).

**Methods.** Total RNA was extracted from cells using an RNA extraction kit (AbGene Ltd) and the concentration was quantified using a spectrophotometer (Wolf Laboratories, York, England, UK). cDNA was synthesized using a first strand synthesis with an oligo<sup>d</sup>T primer (AbGene). PCR was performed using sets of quantitative PCR (Qabsolute) were obtained from AbGene, Surrey, England, UK). Mouse monoclonal antibody ALCAM/CD166 (for immunohistochemistry) was obtained from Novocastra Laboratories (Newcastle upon Tyne, UK). Peroxidase conjugated anti-mouse antibodies were from Sigma (Poole, Dorset, UK) and a biotin universal kit was from Dako, Ltd. (Carpinteria, CA, USA). Bone matrix proteins were prepared from fresh human bone tissues obtained immediately after hip replacement, and collected under the ethical committee approval. Bones were crushed at ice cold temperature and subsequently processed using a BioRuptor instrument (Wolf Laboratories, York, UK), in order to extract matrix proteins.

**Construction of hammerhead ribozyme transgenes targeting the human ALCAM and mammalian expression vector for ALCAM.** Hammerhead ribozymes that specifically target ALCAM, based on the secondary structure of human ALCAM, have been generated as previously described (16-18) (primer details shown in Table I). Anti-ALCAM transgenes were used to transf ect ZR-751 cells which were strongly positive for ALCAM (Figure 1A). Following selection of transfected cells with blasticidin (used at 5 μg/ml) and verification, the following stably transfected cells were established: ALCAM knock-down cells (designated here as ZR751ALCAMrib), plasmid-only control transfection and ALCAM expression, respectively. Stably transfected cells which were negative for ALCAM were transfected with either the control vector or ALCAM expression vector. Stably transfected cells were designated as MDA231pEF/His and MDA231 ALCAMexp, for control transfection and ALCAM expression, respectively.

**RNA preparation and RT-PCR.** Total RNA was extracted from cells using an RNA extraction kit (AbGene Ltd) and the concentration was quantified using a spectrophotometer (Wolf Laboratories, York, England, UK). cDNA was synthesized using a first strand synthesis with an oligo<sup>d</sup>T primer (AbGene). PCR was performed using sets of

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**Table I. Primers used in the present study.**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5'-3')</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALCAMrib1F</td>
<td>CTG CAG CAC TTT TCC ATT CTT GTA CCA TGT CTG ATG AGT CCG TGA GGA</td>
<td>48</td>
</tr>
<tr>
<td>ALCAMrib1R</td>
<td>ACT AGT TAT CCA GTA TGT CAA TTT TCG TCC TCA CGG ACT</td>
<td>39</td>
</tr>
<tr>
<td>ALCAMrib2F</td>
<td>CTG CAG TAC GTC AAG TCG GAA AGG TCT CTG ATG CGT CCG TGA GGA</td>
<td>45</td>
</tr>
<tr>
<td>ALCAMrib2R</td>
<td>ACT AGT ATG GAG ATA CCA TTT TTC GTC TCC ACG GAC T</td>
<td>37</td>
</tr>
<tr>
<td>ALCAMrib3F</td>
<td>CTG CAG CTG ATC TTT GCA TTA CTC TGA TGA GTC CGT GAG GA</td>
<td>41</td>
</tr>
<tr>
<td>ALCAMrib3R</td>
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<td>42</td>
</tr>
<tr>
<td>ALCAMF6</td>
<td>TTA TCA TAC TTT GGC GAT T</td>
<td>19</td>
</tr>
<tr>
<td>ALCAMR6</td>
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<td>20</td>
</tr>
<tr>
<td>GAPDHF8</td>
<td>GCC TGC TTT TAA CTC TGG TA</td>
<td>20</td>
</tr>
<tr>
<td>GAPDRR8</td>
<td>GAC TGT GGT CAT GAG TCC TT</td>
<td>20</td>
</tr>
<tr>
<td>ALCAMExF1</td>
<td>AAT ATG GAA TCC AAG GGG G</td>
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</tr>
<tr>
<td>ALCAMEXR1</td>
<td>GGC TTC AGT TTT GTG ATT GT</td>
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<tr>
<td>ALCAMExF2</td>
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<tr>
<td>ALCAMExR2</td>
<td>GGC TTC AGT TTT GTG ATT G</td>
<td>19</td>
</tr>
</tbody>
</table>
primers (Table I) with the following conditions: 5 min at 95˚C, and then 20 sec at 94˚C-25 s at 56˚C, 50 s at 72˚C for 36 cycles, and finally 72˚C for 7 min. β-actin was amplified and used as a housekeeping control. PCR products were then separated on a 0.8% agarose gel, visualised under UV light, photographed using a Unisavetm camera (Wolf Laboratories) and documented with Photoshop-CS4 (San Jose, CA, USA) software.

In vitro cell growth assay. This was based on a previously reported method (20) Cells were plated into 96-well plated at 2,000 cells/well followed by a period of up to 5 day’s incubation. Cells were fixed in 10% formaldehyde on the day of plating and daily for the subsequent 5 days. 0.5% Crystal violet (w/v) was used to stain cells. Following washing, the stained crystal violet was dissolved with 10% (v/v) acetic acid and the absorbance was determined at a wavelength of 540 nm using an ELx800 spectrophotometer (Wolf Laboratories, York, UK). Absorbance represents the cell number.

Electric cell-substrate impedance sensing (ECIS)-based cell adhesion assay. Two models of ECIS instrument were used: ECIS 9600 for screening and ECIS 1600R for modelling. In both systems, 8W10E arrays which have 10 electrodes in each well were used (Applied Biophysics Inc, NJ, USA) (21-23). Following treatment of the array surface with a cysteine solution, the arrays were incubated with complete medium for 1 h. Respective cells (300,000 per well) in the same volume of medium were added to the arrays and incubated overnight to reach confluence. Baseline electric resistance (without addition of cancer cells) was first recorded, and then cancer cell or control medium was added carefully to the cancer cells. Electrical changes were monitored continuously for up to 24 h. In the 9600 system, the monitoring was at fixed 30 Hz. In the 1600R system, two conditions were recorded: 400 Hz, 4,000 Hz and 40,000 Hz for screening the nature of endothelial changes and 4,000 Hz for fix frequency for cell modelling.

Statistical analysis was carried out using Minitab (version 12) (State College, PA, USA). The Anderson-Darling test was used for normality testing and the Student’s t-test for significance testing.

Results

Expression of ALCAM in breast cancer cell lines and establishing stably transfected sublines. As shown in Figure 1B, MDA MB-463, ZR751 and BT474 were positive, MDA MB-436, MCF-7, MDA MB-468 and BT482 were weakly positive for ALCAM transcripts. In contrast, MDA MB-435, MDA MB-231, BT549 and MDA MB-157 were negative. ZR-751 and MDA MB-231 were selected for knocking down (using ribozyme transgenes) and over-expression, respectively.
Figure 1C shows that transgene-1 and 2 and expression construct-4 were effective in knocking down the ALCAM transcript. Stable transfectants were established from these strains and used for subsequent studies.

Levels of ALCAM expression breast cancer cells directly affect the rate of cell growth in bone matrix proteins. When ALCAM was overexpressed MDA MB-231 cells showed substantially reduced rate of growth in the presence of bone matrix proteins (Figure 2A). This is particularly so when the concentration of BMP was higher than 15%. In contrast, the change of growth pattern was not fully reproduced in ZR-751 cells after knocking down ALCAM (Figure 2B), which may suggest that the incomplete knockdown as seen in Figure 1C may have contributed to this action.

ALCAM expression impacts on the matrix adhesion of breast cancer cells. Overexpression of ALCAM in MDA MB-231 cells resulted in significant reduction of cell adhesion to extracellular matrix, Matrigel (Figure 3A). Using hepatocyte growth factor (HGF) as a stimulus, wild-type and control cells showed a significant increase in the adhesion. However, expression of ALCAM also substantially reduced the adhesion in the cell. In ZR-751 cells, knocking down ALCAM increased the adhesion of the cells to Matrigel (Figure 3B). Using a BMP-coated surface, the same reduction of adhesion is shown as when MDA MB-231 cells overexpress ALCAM (Figure 3C). Using the ECIS method, the reduction of adhesion to culture surface (Figure 3D) and BMP-coated surface (Figure 3E) were similarly observed.

In vitro invasiveness of breast cancer cells is affected by the expression profile of ALCAM. Two matrix models of the in vitro invasiveness were used: Matrigel and Matrigel mixed with BMP. As shown in Figure 4A, overexpression of ALCAM in MDA MB-231 cells resulted in significant
reduction of invasiveness in the Matrigel model. A smaller but nonetheless similar reduction is seen with the Matrigel mixture (Figure 4C). In contrast, knockdown ALCAM from ZR-751 resulted in an increase in invasiveness of invasion into Matrigel (Figure 4B).

Discussion

This study shows that ALCAM expression is different in different breast cancer cell lines. It is further shown that overexpression of ALCAM in breast cancer cells results in marked reduction of cell growth, adhesion to matrix and migration. The opposite has been seen when ALCAM is knocked out of breast cancer cells. Data from the present study support the clinical observation that ALCAM expression in breast cancer may be linked to the likelihood of the tumour developing bone metastasis (15).

The growth of both MDA MB-231 and ZR-751 cells was investigated in a variety of different concentrations of both standard medium and BMP. It was clear that high levels of ALCAM expression (in wild-type ZR-751 and in MDA MB-231ALCAMexp) are associated with cell growth under routine culture conditions and in the presence of BMP. Thus, the presence of ALCAM reduces the growth rate in cancer cells and the absence of ALCAM increases it. This conclusion correlates well with a clinical study where more aggressive fast growing tumors had very low levels of ALCAM (13).

The ability of a cancer cell to adhere to the either a basement membrane of standard medium or of BMP is the first stage in the spread of a cancer cell. As described previously, the ability to adhere to the basement membrane of a blood vessel wall allows extravasation and subsequent intravasation at a distant site. An increase in the ability of a cancer cell to adhere would increase the ability of the cancer to metastasize. The presence of ALCAM is predicted to reduce the ability of cancer cells to adhere to a basement membrane, since the tumors with the lowest levels of ALCAM that metastasised in this study. In performing the adhesion experiments, as well as using standard medium and BMP, the same media were run with HGF added, since this has been shown to increase the ability of cancer cells to adhere. The results obtained from the adhesion experiment showed that MDA MB-231 and ZR-751, when manipulated to have different expression pattern of ALCAM, showed a correlation between the levels of ALCAM and adhesiveness. The same inverse correlation has been observed between the level of ALCAM and invasiveness of breast cancer. These results are in line with observations made in a previous clinical study, in which tumours which subsequently developed bone metastasis tend to have low levels of ALCAM (15).

The present study thus provides new evidence that breast cancer cells expressing low levels of ALCAM are likely to adhere to bone matrix and survive in an environment that also
contain materials obtained from bone matrix. The method by which ALCAM interacts with specific protein(s) from the bone preparation remains to be elucidated further. However, these observations, together with recent reports from clinical studies, point strongly to an inhibitory role of ALCAM in the development of bone metastasis from breast cancer.

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References