Role of ICAM1 in invasion of human breast cancer cells

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We identified previously a region on chromosome 19p13.2 spanning the genes encoding the intercellular adhesion molecules (ICAM), ICAM1, ICAM4 and ICAM5 as a breast cancer susceptibility locus. Genetic variants in this region were also associated with indicators of disease severity, including higher rates of metastases to other organs. Based on this association, we set out to explore the role of ICAM1 in proliferation and invasion of human breast cancer cells. We observed that ICAM1 downregulation at the mRNA and protein levels led to a strong suppression of human breast cell invasion through a matrigel matrix. Under the same conditions, no significant effect on cell proliferation in vitro was seen. Incubation of cells with an antibody against ICAM1 blocked invasion of the highly metastatic MDA-MB-435 cell line in a dose-dependent manner without affecting cell migration. We also demonstrated that the level of ICAM1 protein expression on the cell surface positively correlated with metastatic potential of five human breast cancer cell lines and that ICAM1 mRNA levels were elevated in breast tumor compared with adjacent normal tissue. These results corroborate our previous genetic finding that variations in the ICAM region are associated with the occurrence of metastases and establish a causal role of ICAM1 in invasion of metastatic human breast carcinoma cell lines.

Introduction

Intercellular adhesion molecule 1 (ICAM1) is a cell surface glycoprotein in the immunoglobulin superfamily. ICAM1 is expressed at a low basal level in fibroblasts, leukocytes, keratinocytes, endothelial and epithelial cells but is upregulated in response to a variety of inflammatory mediators (1). It is associated with a number of inflammatory and immune responses, as well as with epithelial tumorigenesis (2). The published evidence is controversial on whether upregulation or inhibition of ICAM1 expression contributes to human tumor progression, particularly of breast cancer. Immunohistochemical analysis of certain colorectal and breast cancer tissues indicated low expression levels of ICAM1 (3–5). This led to the conclusion that ICAM1 may be involved in tumor suppression via an immuno-surveillance mechanism (6–8). On the other hand, elevated expression of this protein has been reported in gastric, pancreatic and breast cancer tissues, with highest levels in samples from patients with metastases (9–16).

ICAM1 can act as an adhesion molecule, but can also elicit a signaling response which could enhance a metastatic phenotype (1,17–19). There is substantial evidence for the potential involvement of ICAM1 in metastasis of some tumors. Inhibition of ICAM1 has been shown to block invasion of lung cancer cells in vitro (20). A report of ICAM1 inhibition in melanoma cells using antisense oligonucleotides showed decreased metastases to lung (21). Multiple correlation studies have demonstrated an increase in soluble serum ICAM1 fraction in breast cancer patients, but it is not clear whether this soluble form originates from endothelial or epithelial cells (11,12). Thus, a direct evidence for ICAM1 involvement in breast cancer progression is not yet available.

We have reported previously that DNA variants in the ICAM gene region on chromosome 19p13.2 are associated with susceptibility to breast cancer (22). Using high-density SNP mapping we showed that the extent of association spans 20 kb and includes the intercellular adhesion molecule genes ICAM1, ICAM4 and ICAM5. Based on expression patterns, ICAM1 was proposed as the most likely candidate for the observed association. Genetic variants in the region were also associated with disease progression, and patients carrying the susceptibility alleles showed increased incidence of organ metastases. In the present study, we explored the role of ICAM1 in proliferation and invasion of breast tumor cells. We profiled ICAM1 expression in human cancer cell lines and breast tumor tissues and examined the effect of suppressing ICAM1 by small-interfering RNA (siRNA)-mediated inhibition of expression and by antibody-mediated blockade of protein function. This is the first study to report that ICAM1 is likely to play a major role in invasion of cancerous cells, and therefore in growth and metastasis of breast tumors.

Materials and methods

Cell culture and siRNA-mediated gene silencing

MDA-MB-231 and MDA-MB-435 breast cancer cell lines were obtained from the Developmental Therapeutics Program, NCI/NIH (Frederick, MD) and were cultured according to ATCC, Manassas, VA) and were cultured according to ATCC recommendations. Normal human dermal fibroblasts (NHDF) were obtained from BioWhittaker (Walkersville, MD) and cultured according to the manufacturer’s instructions. Small-inhibitory RNA duplexes were designed according to the guidelines of Elbashir et al. (23) and were synthesized by Dharmacon Research Inc. (Lafayette, CO). Oligonucleotide siRNA sequences for human ICAM1 were designed as follows: siICAM1-1, 5'-AACCAACCCGGAAGGGUGAUGA; siICAM1-2, 5'-AAAGCACCACAUGUGCUGUAUCU; siICAM1-3, 5'-AAGAUCACCAUGGACCAAU; siICAM1-4, 5'-AUCUGUACUCGAGUUCAUGA. A control siRNA siGL2 (5'-AUCUGUACUGGCAUUCUGA), which is non-homologous to any human sequence, was obtained from Dharmacon. Additional control siRNA were designed to target human ICAM5.
and RAD21 mRNA (siICAM5, 5'-UAAUGACCGAGACGAGCAA; siRAD21, 5'-AAGGAGUGGAGAUGCAGACAA). Cells were plated in 6-well culture dishes to achieve 70–85% confluency on the following day. A mixture of siRNA (35–150 nM) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a ratio of 1:3 (µg/µl) in a total volume of 1.25 ml OptiMEM I (Invitrogen) was incubated for 30 min at room temperature to form liposomes. This mixture was then added to cells that have been prewashed with OptiMEM I medium. After incubating for 5 h at 37°C, 1.25 ml of standard medium containing 20% FCS was added. The final concentration of siRNA during the overnight incubation was 18–75 nM. The cells were incubated for an additional 16 h at 37°C, washed with complete medium and replated either on 96-well plates for determination of proliferation or on chambered glass slides for an apoptosis assay. For preparation of mRNA or determination of potential invasion, cells were replated on 6-well plates to achieve 70–90% confluency, 2–3 days post-transfection.

cDNA preparation and quantitative gene expression (QGE) by MassARRAY

Total RNA samples from human normal breast, lung, heart and skin tissues were purchased from Ambion (Austin, TX) and used for cDNA synthesis. To assess cellular expression of mRNA, 50 × 10⁶ cells were collected and mRNA isolated using Dynalbead mRNA Direct (Dynal, Oslo, Norway) according to manufacturer’s protocols. Frozen human clinical breast tumor samples were purchased from ProteoGenex (Los Angeles, CA). Total RNA from tissues was extracted using TRIzol (Invitrogen, Carlsbad, CA). For siRNA experiments, cells were harvested on day 2 post-transfection with siRNA, and total RNA extracted using TRIzol. cDNA was prepared using random hexamers or oligo-dT primers and Superscript II reverse transcriptase (Invitrogen) and these preparations were pooled. Levels of transcripts were assessed using quantitative RT–PCR and mass spectrometry [quantitative gene expression (QGE) by MassARRAY assay, Sequenom, San Diego, CA]. The competitive PCR step of QGE by MassARRAY assay was described elsewhere (24). Levels of gene-specific mRNA were normalized against levels of gamma 1 actin (ACTG1) mRNA by dividing the observed transcript concentration of each target gene (ICAM1 or ICAM5) by the observed transcript concentration of ACTG1 for each respective sample. Normalized data are expressed as ratios. All QGE oligonucleotides used in this study are shown in Table 1. QGE analysis included triplicate experiments with quadruplet spotting of reaction products onto SpectroCHIPs (Sequenom).

Protein expression analysis by flow cytometry

Cells were removed from tissue culture plates using Cellstripper, a non-enzymatic cell dissociation reagent containing a proprietory mixture of chelators (Mediatech, Herndon, VA). Next, cells were brought to a concentration enzymatic cell dissociation reagent containing a proprietory mixture of chelators (Mediatech, Herndon, VA). Next, cells were brought to a concentration of 10⁵ cells per 100 µl of PBS containing 1% FCS, followed by incubation with monocolonal anti-human ICAM1 antibody (2 mg/ml, clone BBIG-II (11C81) IgG1, R&D Systems, MN) or isotype control mouse IgG1 antibody (2 mg/ml, Jackson ImmunoResearch Labs, West Grove, PA) for 30 min at 4°C. Cells were washed twice in PBS-1% FCS and then incubated with a secondary goat anti-mouse antibody conjugated with phycoerythrin (Jackson ImmunoResearch). Recombinant human ICAM1 (rhICAM1, BD Biosciences, San Jose, CA) was used to determine the specificity of the anti-ICAM1 staining in each of the cell lines assayed. Flow cytometry was performed using a Guava personal flow cytometer (Hayward, CA). For cells treated with siRNA, ICAM1 staining was performed 2–3 days post-transfection.

Cell proliferation and apoptosis assays

Cell proliferation was measured using the WST-1 assay kit (Roche Diagnostics, Indianapolis, IN) at designated time points, and relative proliferation calculated by normalizing to day 1 values. Experiments were performed at least three times. Apoptosis was measured on day 2 using the Vybrant apoptosis assay kit 3 (Molecular Probes, Eugene, OR) as directed by the manufacturer.

Boyden chamber invasion and migration assays

Invasion assays were performed using porous (8 µm) filters coated with growth-factor reduced matrigel (BD Biosciences, San Diego, CA) to occlude the pores. The lower chamber contained 750 µl of conditioned medium from a 24 h confluent culture of the corresponding cells. A total of 30 000–50 000 MDA-MB-231 or MDA-MB-435 cells per well, were added to the upper chamber in 500 µl of serum-free medium containing 0.1% bovine serum albumin (Sigma-Aldrich, St Louis, MO). After 18–18 h incubation, cells were briefly washed with PBS, fixed in 2% glutaraldehyde for 10 min and stained in 0.2% crystal violet. Chambers were photographed before and after the upper chambers were scraped to remove cells that did not invade. The photographed cells were counted to quantify percent invasion. ‘No treatment’ siRNA control samples were mock transfected in the absence of siRNA. The total cell numbers for both chambers were the same in control (no treatment, siGL2 or IgG1) and experimental (siICAM or anti-ICAM1) wells. The migration assays were performed in a similar fashion except that the filters were not coated with matrigel and therefore the pores were open for cell passage. For ICAM1 antibody experiments, migration and invasion assays were performed after pretreatment of cells with 2 or 10 µg/ml antibody for 30 min at 4°C.

Results

ICAM1 expression is elevated in several cancer cell lines and tissues

We first characterized ICAM1 mRNA expression in a panel of human cancer cell lines using semi-quantitative RT–PCR (data

<table>
<thead>
<tr>
<th>Gene</th>
<th>PCR primers</th>
<th>MassEXTEND primer</th>
<th>Competitor*</th>
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<td>ICAM1</td>
<td>F: ACGTTGGATGACCAAGGGAGGCACAC</td>
<td>CGTGAAATGTCCTTCC</td>
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<tr>
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<td>ATGTGCAAGGTGCTTCTTGATGAGG</td>
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<tr>
<td>R: ACGTTGGATGACCAAGGGAGGCACAC</td>
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*Artificial polymorphisms in competitor sequences are underlined.
Results obtained were consistent with data from the NCI Developmental Therapeutics Program repository (http://dtp.nci.nih.gov/mtweb/), which show high expression of ICAM1 in cell lines derived from melanoma, ovarian, renal, prostate, leukemia, lung cancer and breast cancer cells. We further quantitated cell surface expression of ICAM1 protein in five breast cancer cell lines using an antibody against the extracellular N-terminal region of this molecule (Figure 1A, first five cell lines, left to right). The ICAM1 protein expression levels positively correlated with the reported metastatic potential of each of the cell lines. The most metastatic MDA-MB-435 cells showed the highest level of expression (25). The expression levels in a normal skin fibroblast line and in the metastatic lines NCI-H460, A375 and PC3 derived from lung, melanoma and prostate, respectively, are shown for comparison (Figure 1A). To confirm whether expression of ICAM1 correlates with certain pathological phenotypes in clinical breast tumor samples, we performed QGE by MassARRAY on patient-matched normal and tumor adjacent tissue pairs. Analysis of these human tissue showed a trend toward elevated ICAM1 mRNA levels in the tumor versus the adjacent normal tissue. The stage 3B infiltrating ductal carcinoma tissue had the greatest mRNA expression difference of 15-fold relative to the normal adjacent breast tissue (Figure 1B). These data confirm reports by others (9,14,15,26,27) and stress the potential involvement of ICAM1 in breast tumorigenesis. We also observed low levels of ICAM1 mRNA expression in most normal tissues except lung (Figure 1B). It should be noted that possible monocytic infiltration of clinical samples could complicate proper analysis of ICAM1 expression in frozen tissue specimens. The high expression in the lung could be explained by the fact that ICAM1 is the major receptor for rhinoviruses in lung epithelial cells and its expression in this tissue is very sensitive to the infection state of the individual from which the sample was derived (28,29).

**Suppression of ICAM1 in breast cancer cells by RNAi**

To investigate whether ICAM1 could serve as a therapeutic target in breast cancer, we used synthetic siRNA duplexes to deplete ICAM1 expression in three human breast cancer cell lines: the non-metastatic cell line MCF-7, the metastatic cell line MDA-MB-231 and the highly metastatic cell line MDA-MB-435. We designed four siRNA sequences targeting the coding region of human ICAM1 mRNA. These were transfected into MCF-7 and MDA-MB-231 cells. In some experiments, we included siRNA against human ICAM5 (siICAM5) and a non-human siRNA (siGL2) as control. Two days after transfection, cell lysates were harvested and cDNA prepared and analyzed for ICAM1 gene expression by QGE. Figure 2A

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**Fig. 1.** ICAM1 expression in normal and cancer cell lines and tissues. (A) Quantitation of ICAM1 protein expression in human cell lines by flow cytometry. Values plotted were obtained from the background-subtracted mean fluorescence intensity (MFI) of at least two independent antibody staining experiments and normalized to the MFI of MDA-MB-231 cells. MDA-MB-231 and MDA-MB-435 data are from four independent experiments. Data shown represent means ± SD. (B) Quantitation of ICAM1 mRNA expression in human tissues using QGE by MassARRAY. Expression was normalized to that of gamma 1 actin (ACTG1) as described in the Materials and methods section. Data shown represent means of triplicate experiments ± SD.
Fig. 2. Suppression of ICAM1 mRNA and protein levels by siRNA. (A) ICAM1 mRNA expression in MCF-7 and MDA-MD-231 cells harvested 2 days post-siRNA-transfection. Expression was quantitated using QGE by MassARRAY. The homologous ICAM5 mRNA was analyzed in parallel to determine target specificity of the siRNA. Values were normalized to ACTG1. NT, lipofectamine only. (B) ICAM1 protein expression in MCF-7 and MDA-MD-231 cells harvested 3 days post-siRNA-transfection. Expression was quantitated by flow cytometry and expressed as background-subtracted MFI. (C) Flow cytometry analysis of ICAM1 protein expression in MDA-MB-231 cells. Results of a representative experiment are shown.
shows the results of these experiments using individual siRNA duplexes. ICAM1 gene expression was significantly suppressed by siICAM1-1. ICAM1 mRNA levels were reduced by ~75% in MCF-7 cells and >90% in MDA-MB-231 cells as compared with the cells treated with siICAM5 or siGL2. The inhibitory effect of siICAM1-1 was likely to be specific because it did not cause a non-specific downregulation of the human homologous ICAM5 gene (Figure 2A). Expression of human RAD21 and the housekeeping genes ACTG1 and HMBS was also not affected (data not shown). SiICAM1-2 down-regulated ICAM1 in both cell lines but upregulated ICAM5 in MDA-MB-231 cells. SiICAM1-3 inhibited ICAM1 expression in MCF-7 slightly but had no effect on MDA-MB-231 cells where, interestingly, ICAM5 expression was downregulated. SiICAM1-4 strongly inhibited ICAM1 in MDA-MB-231 cells but had no effect on MCF-7 cells. At the same time, siICAM1-4 significantly upregulated ICAM5 in MCF-7 cells while inhibiting this gene in MDA-MB-231 cells. It is likely that siICAM1-2, siICAM1-3 and siICAM1-4, in addition to targeting ICAM1, also caused off-target effects. Therefore, we concluded that the inhibitory effect of siICAM1-2, siICAM1-3 and siICAM1-4 on ICAM1 gene expression in MDA-MB-231 as well as MCF-7 cells was non-specific, whereas the effect of siICAM1-1 in both cell lines was specific. In MDA-MB-435 cells, siICAM1-1 inhibited ICAM1 gene expression by 75 ± 12%. Similar results were also obtained in MDA-MB-435 cells for siICAM1-3 and siICAM1-4 (data not shown). Based on these data, in all subsequent experiments, we selected siICAM1-1 for the specific downregulation of ICAM1 expression.

Next, we performed an analysis of cell surface ICAM1 antibody staining in transfected MCF-7 and MDA-MB-231 cells. Figure 2B and C demonstrates the mean fluorescence intensity (MFI) values of anti-ICAM1 antibody staining from a representative experiment. The specificity of the test system was established using untreated cells with and without pre-incubation of antibodies with recombinant human ICAM1 (rhICAM1). Pre-incubation with rhICAM1 almost completely abolished immunofluorescence in experiments with both cell lines. It should be mentioned that rhICAM1 reduced the fluorescence to the same level as that of the isotype control IgG1 plus secondary antibody (data not shown). Therefore, we concluded that the system is appropriately specific. Significant downregulation of ICAM1 protein expression in both cell lines after siICAM1-1 treatment was demonstrated. This effect was more pronounced in the metastatic MDA-MB-231 cell line. MFI values from three independent experiments showed an average suppression of ICAM1 surface protein of 60 ± 12% in MDA-MB-231 cells and 42 ± 9% in MCF-7 cells.

**Cell proliferation is not affected by the specific ICAM1-1 siRNA**

We examined whether a suppression of ICAM1 affects proliferation of breast cancer cell lines in vitro. MDA-MB-231 and MDA-MB-435 cells transfected with siICAM1-1 siRNA grew at a rate similar to cells transfected with control siGL2 (Figure 3). Therefore, selective inhibition of ICAM1 did not affect proliferation of these cells growing under regular tissue culture conditions. Similar results were obtained for MCF-7 breast cells also indicating that ICAM1 is not required for the proliferation of these cells in culture (data not shown). Consistent with the absence of an effect of siICAM1-1 on cell proliferation, staining of cells for the early apoptotic

**Inhibition of ICAM1 reduces human breast cancer cell invasion in vitro**

To determine whether the loss of ICAM1 expression can affect the ability of cells to invade in vitro we used the cell line MDA-MB-435, which was derived from a breast cancer with high metastatic potential and had high expression levels of ICAM1. We compared the ability of these cells to invade the matrigel after transfection with specific siICAM1-1, non-specific siICAM1-3 and siICAM1-4, and control siGL2. Only treatment with siICAM1-1 effectively and significantly inhibited invasion (Figure 4A). Non-specific ICAM1 siRNA species (siICAM1-3 and siICAM1-4) and control siGL2 did not influence invasion as compared with mock-transfected cells (Figure 4A). Quantitative estimates of invasion inhibition by siICAM1-1 were derived from analysis of microphotographs based on three independent experiments. SiICAM1-1 alone inhibited invasion by 85 ± 10% relative to the siGL2 control (0% inhibition). Given the fact that the suppression of ICAM1 protein expression was <60% in the majority of cells and that MDA-MB-435 cells express very high levels of ICAM1, it was
surprising to detect such a strong inhibition of invasion. This suggests that for a particular cell type, a threshold level of ICAM1 protein is required for invasion to occur.

Further proof of ICAM1 playing a role in invasion was obtained from the antibody blocking experiments. Incubation with 2 μg/ml anti-ICAM1 antibody led to 10% inhibition of invasion (data not shown). Increasing antibody concentration to 10 μg/ml caused almost complete inhibition of invasion similar to the effect of siCAM1-1 siRNA transfection (Figure 4B). Control IgG1 had no effect on invasion of MDA-MB-435 cells, thus indicating the specificity of the result. The following quantitative data were calculated from two independent experiments with MDA-MB-435 cells: at 2 μg/ml, anti-ICAM1 antibody inhibited invasion by 15 ± 6%, whereas at 10 μg/ml, invasion was inhibited by 90 ± 4%, relative to the IgG1 control. An unexpected finding was that targeting ICAM1 with siRNA or antibody did not affect cell migration (data not shown). Thus, ICAM1 seems to play a causal role in breast cancer cell invasion but does not seem to be involved in cell migration in vitro.

Discussion

In a recently published genome-wide association study using DNA samples from clinically diagnosed breast cancer cases and matched controls, we have reported that a region on chromosome 19p13.2 containing the genes ICAM1, ICAM4 and ICAM5 influences breast and prostate cancer risk (22). In the same study, the susceptibility allele of the variant rs281439 in the 5' flanking region of ICAM5 also correlated with a higher rate of metastases to other organs (P = 0.003). A similar effect was observed for the non-synonymous SNP in the C-terminal immunoglobulin domain of ICAM1 (K469E) where 7% of the individuals homozygous for the susceptibility (K) allele had organ metastases as opposed to 0% of the patients homozygous for the protective (E) allele (P = 0.1, data not shown). In the present study, we provide biological evidence that ICAM1 may be involved in metastasis of human breast cancer.

We started our functional studies by analyzing ICAM1 expression in a number of human tissues and cell lines. We found that ICAM1 expression levels positively correlated with
ICAM1 activation has been shown to increase expression interaction with fibrinogen in the matrigel (27), thus enhancing invasive signal, ICAM1 may also stimulate signaling via its made by others (47). In addition to the MUC1-mediated pro-calcium ion-based proinvasive signal within tumor cells. This contributes to the formation of cell clusters followed by a case-control study (22) is a candidate responsible for functional changes. The amino acid residue 469 is located in the extracellular portion of the protein, and therefore it is possible that the variant leads to changes in ICAM1 interactions with proteins in the extracellular environment. This or other variants in ICAM1 may result in a higher affinity ligand or receptor and thereby enhance the metastatic phenotype (gain-of-function mechanism). The available expression data also advocate that protein upregulation may contribute to this phenotype. Therefore, relevant functional SNPs may also reside in regulatory elements of the ICAM1 gene. Further analysis of this gene region in parallel with functional experiments should facilitate elucidation of the precise molecular mechanisms, by which ICAM1 influences tumor metastasis.

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Conflict of Interest statement: Of the co-authors, P.O., A.B., S.K. and M.F.D. declare that they hold stock in Sequenom, Inc., the maker of MassARRAY product, and are currently conducting research sponsored by this company.

References