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Suppression of Metastasis in Human Breast Carcinoma MDA-MB-435 Cells after Transfection with the Metastasis Suppressor Gene, KiSS-I

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Abstract

Based on the observation that chromosome 1q deletions are not infrequent in late-stage human breast carcinomas, we tested whether the recently discovered human melanoma metastasis suppressor gene, KiSS-I, which maps to chromosome 1q32-q41, could suppress metastasis of the human breast carcinoma cell line MDA-MB-435. Parental, vector-only transfected and KiSS-I transfected clones were injected into the mammary fat pads of athymic nude mice and assessed for tumor growth and spontaneous metastasis to regional lymph nodes and lungs. Expression of KiSS-I reduced metastatic potential by 95% compared to control cells but did not suppress tumorigenicity. Metastasis suppression correlated with a decreased clonogenicity in soft (0.3%) and hard (0.9%) agar. Although the overall rate of cell adhesion to extracellular matrix components was unaffected, KiSS-I transfected spread on immobilized type-IV collagen more rapidly than did control populations. Invasion and motility were unaffected by KiSS-I. Based on the predicted structure of the KiSS-I protein, our results implicate a mechanism whereby KiSS-I regulates events downstream of cell-matrix adhesion, perhaps involving cytoskeletal reorganization. In addition to its already described role in melanoma, our results show that KiSS-I also functions as a metastasis suppressor gene in at least some human breast cancers.

Introduction

KiSS-I was identified as a human melanoma metastasis suppressor gene using subtractive hybridization between the metastatic human melanoma cell line C8I61 and nonmetastatic variants generated after microcell-mediated transfer of chromosome 6 into C8I61 (1). Transfection of KiSS-I into metastatic human melanoma cell lines C8I61 and MeIUSo suppressed metastasis in athymic nude mice by 50—95% (1, 2). The KiSS-I gene maps to chromosome 1 bands q32—q41 and predicts a hydrophilic, 164-amino acid protein with a proline-rich domain suggestive of an SH3 ligand and a putative protein kinase C-a phosphorylation site. These domains within KiSS-I suggest that KiSS-I could be involved in the signal transduction pathway.

The purpose of this study was to determine whether KiSS-I might function as a metastasis suppressor in another tumor type. Breast cancer is the most common cancer among women in North America and western Europe and is the second leading cause of female cancer deaths in the United States (3). Like most solid tumors, metastatic disease rather than the primary tumor itself is responsible for death. The rationale for studying KiSS-I in human breast cancer is based on studies showing loss of heterozygosity for regions of chromosome 1q (4—6). Involvement of chromosome 1q is not frequent in most cancer types; therefore, the frequency of loss of heterozygosity suggested that a gene involved in breast cancer progression was encoded therein.

Here we report a test of that hypothesis using transfection of the metastatic human breast carcinoma cell line MDA-MB-435 with KiSS-I cDNA. Transfectants formed significantly fewer pulmonary and regional lymph node metastases than did control or neo-transfected clones. These results suggest that KiSS-I can function as a breast cancer metastasis suppressor gene and may indeed be involved in the progression of human breast cancer toward malignancy.

Materials and Methods

Cell Lines and Culture. MDA-MB-435 and MDA-MB-231 are estrogen receptor- and progesterone receptor-negative, metastatic, ductal breast carcinoma cell lines (7). Both cell lines form tumors when injected into the mammary fat pads of nude mice, and macroscopic metastases to lungs and regional lymph nodes can be identified 10—18 weeks postinoculation. However, MDA-MB-435 forms more metastases in a greater percentage of athymic nude mice than do the MDA-MB-231 cells. Therefore, the former were chosen for transfection studies. MDA-MB-435 cells were the generous gift of Dr. Janet Price (University of Texas M. D. Anderson Cancer Center, Houston, TX). MDA-MB-231 cultures were kindly provided by Dr. Robert Gillies (University of Arizona Cancer Center, Tucson, AZ).

Both human breast cancer cell lines were maintained in DMEM:F12 medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (cDMEM:F12; Atlanta Biologicals) and no antibiotics. The neomycin-resistant KiSS-I transfectants were maintained in cDMEM:F12 containing 500 μg/ml G418 (Life Technologies, Inc., Gaithersburg, MD). All cell lines were free of Mycoplasma sp. contamination as determined by a PCR-based test (PanVera, Madison, WI).

Transfection of KiSS-I. The construction of pcDNA3-KiSS-I expression vector was described previously (1). pcDNA3-KiSS-I vector was transfected into MDA-MB-435 cells using Lipofectin (Life Technologies, Inc.) according to the manufacturer’s instructions. pcDNA3 vector only was also transfected as a control. G418-resistant clones were isolated by growth in selective medium. Uncloned, stable transfected cells (MDA-MB-435-KiSS-I mix) were cloned by limiting dilution (MDA-MB-435-KiSS-I cl.1, MDA-MB-435-KiSS-I cl.2, MDA-MB-435-KiSS-I cl.3, and MDA-MB-435-KiSS-I cl.7).

Northern Blotting. RNA expression analysis was done as described previously. Total RNA from cell cultures and tumor tissues was isolated using a RNeasy kit® (Qiagen, Chatsworth, CA). Poly(A)+-enriched mRNA was isolated using a Micro FastTrack kit® (Invitrogen, San Diego, CA). Total RNA (20 μg) or poly(A)+-enriched RNA (2.5 μg) was size-fractionated in a 1% agarose gel containing 2.2 M formaldehyde. After transfer and UV cross-linking, the nylon membranes were probed with a full-length 32P-labeled KiSS-I cDNA.

Migration and Adhesion Assays. Migration was measured by the wound healing method as described previously (8). Briefly, breast carcinoma cells (4 × 105) were plated onto 6-well plates in triplicate and allowed to grow until 80% confluence. The monolayer was scraped with a Teflon-coated plastic cell scraper (Fisher Scientific, Pittsburgh, PA), and the detached cells were removed by washing three times with CMF-DPBS. The remaining adherent cells were incubated in DMEM:F12 containing 0.5% fetal bovine serum for 24—48 h. Photographs of the edge were used to count the migration of cells into the exfoliated space. Adhesion was measured in a 96-well plate assay. Briefly, wells were coated

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The abbreviations used are: poly(A)+, polyadenylate; CMF-DPBS, calcium- and magnesium-free Dulbecco’s PBS.

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with mouse laminin, fibronectin, or collagen type IV (Collaborative Biomedical Product, Bedford, MA) at a concentration of 5 μg/cm² for 1 h at room temperature. Murine adhesion molecules were used, because this is the environment onto which cells would have to adhere in vivo. The wells were then rinsed with CMF-DPBS before preblocking with a solution of DMEM:F12 containing 1% BSA for 1 h at 37°C. After removing the supernatant, cells (4 × 10⁵) suspended in adhesion medium (DMEM:F12 supplemented with 0.5% BSA and 25 μM HEPES) were dispensed into each well, incubated at 37°C for varying times, and gently washed three times with CMF-DPBS. The remaining adherent cells were quantified by measuring acid-phosphatase (9).

To semiquantify the spreading results, the percentage of spread/flattened cells per random high-power field were determined.

Photographs of cells at various times after placement onto the matrices were taken to compare spreading. Tissue culture plates (24 well) were coated with 5 μg/cm² mouse laminin, fibronectin, or collagen type IV as described above. Cells (2 × 10⁶) were suspended in adhesion medium and dispensed into each well before incubation at 37°C for 3 h. Unattached cells were removed by washing with CMF-DPBS.

Clonogenic Soft Agar Assays. Colonization in soft agar was performed as described previously (10), with minor modifications. A layer of cDMEM:F12 with 0.5% agarose was set into 6-well plates. A suspension of cells (10⁵-10⁶) in 0.3% agarose was overlaid onto the basal layer. A similar experiment was performed using hard agar (0.9%) as an upper layer, because this parameter has been shown to be more closely related to metastatic efficiency than soft agar cloning (10). The number of colonies was determined 20–40 days after plating.

Metastasis Assays. Cells (10⁶) were injected into the subaxillary mammary fat pads of 4–6-week-old female athymic nude mice (7–10 mice/group; Harlan Sprague Dawley, Madison, WI). Mice were maintained under the guidelines of the NIH and The Pennsylvania State University College of Medicine. All protocols were approved and monitored by the Institutional Animal Care and Use Committee. Food and water were provided ad libitum. When the mean tumor diameter (square root of the product of orthogonal measurements) reached 1.0–1.3 cm, primary tumors were surgically removed under Ketaset-Rompun anesthesia. Mice were then maintained for an additional 4 weeks to allow further growth of lung metastases. After euthanasia, all organs were checked for metastasis. Visible lung metastases were counted in fixed tissues (neutral-buffered formalin:Bouin’s fixative, 5:1) with the aid of a dissecting microscope as described previously (11).

Statistical Analysis. The number of lung metastases in KISS-I transfectants and control cells (parental and vector-only transfectants) was compared using a Kruskal-Wallis ANOVA of ranks procedure. The percentage of spread/flattened cells observed by H&E staining (data not shown). Of parental and neo transfectants. These findings, however, could not preclude the action of KiSS-I in human MDA-MB-435 breast carcinoma cells significantly reside in the same distribution (95% confidence interval), and the mean KiSS-I expression in MDA-MB-435-KiSS-I transfectant tumors was significantly increased (P < 0.05) compared to parental tumors.

Results

Expression of the KiSS-I transcript in MDA-MB-435 and MDA-MB-231 cells was measured by Northern blot (Fig. 1A). MDA-MB-435 cells are reportedly less invasive in vitro than MDA-MB-231 cells but are significantly more metastatic (7). MDA-MB-435 cells do not express the KiSS-I transcript (1.0 kb), but MDA-MB-231 cells do. Based on their relative KiSS-I expression and metastatic potentials, MDA-MB-435 cells were chosen for subsequent studies.

Full-length KISS-I cDNA was subcloned into the pcDNA3 constitutive expression vector and transfected into MDA-MB-435 cells. Concomitantly, vector without insert was transfected as a negative control. Several single-cell clones were randomly selected after limiting dilution, and the expression of KISS-I was confirmed by Northern blot (Fig. 1B). Transfectants and matched controls were inoculated into the subaxillary mammary fat pads of 4–6-week-old female athymic nude mice. Tumors were measured weekly thereafter to assess the growth rate. Once the mean tumor diameter reached ~1 cm, tumors were surgically removed. One month later, mice were euthanized, and metastases were quantified. Primary tumor removal was done to minimize the effect of different growth rates for clonal populations and to allow micrometastases time to develop into macroscopic lesions. Local tumors were removed from MDA-MB-435-KiSS-I and MDA-MB-435-KiSS-I mix-, MDA-MB-435-KiSS-I cl.1-, MDA-MB-435-KiSS-I cl.2-, MDA-MB-435-KiSS-I cl.3-, and MDA-MB-435-KiSS-I cl.7-injected mice 49, 49, 69, 106, 106, and 53 days after injection, respectively.

Examination of H&E-stained sections of parental MDA-MB-435 and KiSS-I transfected tumors was also done. Both tumor types exhibited characteristics of poorly differentiated invasive adenocarcinoma (data not shown), but we found no consistent histological properties that distinguished KiSS-I transfectants from parental cells.

Tumorigenic and metastatic properties are depicted in Table 1 and Fig. 2. All cells formed tumors; however, the number of macroscopic lung metastases in animals injected with the KiSS-I transfectants was significantly (P < 0.05) decreased. The KiSS-I transcript (1.3 kb) was still expressed in tumor tissues (Fig. 1B), showing that tumor growth was not due to loss of the vector. Parental MDA-MB-435 cells formed an average of 26 lung metastases/mouse. Two vector-only transfectants formed 9 and 18 lung metastases, respectively. The KiSS-I transfectants formed between 1 and 5 lung metastases/mouse. These metastases were extremely small compared to those found in the parental cells or in vector-only transfectants. The presence of additional microscopic metastases in random lung sections was not observed by H&E staining (data not shown). Of parental and neo transfectants, only 1 mouse had no metastasis (1 of 24 mice, 4.2%), whereas 12 of 24 mice (50%) developed > 10 lung metastases. In KiSS-I transfectants, 12 of 39 mice (30.8%) developed no lung metastases, and only 2 of 39 mice (5.1%) had > 10 metastases/animal. In all KiSS-I transfectants, metastasis to lung was suppressed by at least 50%. Likewise, the incidence of regional lymph node metastasis was reduced. These results clearly demonstrate that the expression of KiSS-I in human MDA-MB-435 breast carcinoma cells significantly suppresses metastatic ability in athymic nude mice.

The mechanism by which KiSS-I suppresses metastasis in melanoma cells has not been determined. Neither adhesion to extracellular matrices nor invasiveness in vitro or through reconstituted basement membranes using in vitro assays is altered in melanoma KiSS-I transfectants. These findings, however, could not preclude the action of KiSS-I at these steps in breast cancer metastasis. KiSS-I transfectants are
KiSS-I suppresses metastasis of MDA-MB-435 cells from the mammary fat pad in athymic nude mice.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumorigenicity</th>
<th>Lung metastases Incidence</th>
<th>Mean ± SEM</th>
<th>Median (range)</th>
<th>Lymph node metastases Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-435</td>
<td>8/8</td>
<td>8/8</td>
<td>26 ± 18</td>
<td>8.5 (3–153)</td>
<td>5/8</td>
</tr>
<tr>
<td>Vector 1</td>
<td>8/8</td>
<td>8/8</td>
<td>18 ± 8</td>
<td>10.5 (3–71)</td>
<td>4/8</td>
</tr>
<tr>
<td>Vector 2</td>
<td>8/8</td>
<td>7/8</td>
<td>9 ± 4</td>
<td>5.5 (0–35)</td>
<td>5/8</td>
</tr>
<tr>
<td>KiSS-1/mix</td>
<td>8/8</td>
<td>4/8</td>
<td>1 ± 1</td>
<td>0.5 (0–6)</td>
<td>2/8</td>
</tr>
<tr>
<td>KiSS-1/cl.1</td>
<td>8/8</td>
<td>5 ± 2</td>
<td>2.5 (0–23)</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>KiSS-1/cl.2</td>
<td>8/8</td>
<td>2 ± 1</td>
<td>1.5 (0–7)</td>
<td>0/8</td>
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<tr>
<td>KiSS-1/cl.3</td>
<td>8/8</td>
<td>1 ± 0.4</td>
<td>0.5 (0–2)</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>KiSS-1/cl.7</td>
<td>7/7</td>
<td>3 ± 1</td>
<td>3.0 (1–10)</td>
<td>0/8</td>
<td></td>
</tr>
</tbody>
</table>

a Cells (10⁶) were injected into the subaxillary mammary fat pads of 4–6-week-old female athymic nude mice. When the mean tumor diameter reached 1.0–1.3 cm, primary tumors were surgically removed. Metastases were quantified 4 weeks later.
b Number of mice with metastases/total number of mice injected.
c The pcDNA3 vector without an insert. KiSS-1/mix is the uncloned population of pcDNA3-KiSS-1 transfectants.
d Significantly different (P < 0.05) from parental cells or vector-only transfectants by Kruskal-Wallis ANOVA.

As invasive as parental MDA-MB-435 cells in vivo (data not shown); therefore, in vitro assays were not done. Likewise, significant differences in migration were not observed (data not shown).

Although the kinetics of adhesion to tissue culture plates coated with murine collagen type IV, laminin, or fibronectin were unchanged in KiSS-I transfectants (i.e., the number of cells attached throughout the adhesion assay was identical to that of untransfected controls; data not shown), the rate of spreading (i.e., flattened morphology) on type-IV collagen was different (Fig. 3). Parental and vector-only transfectant cells were still predominantly rounded 3 h after plating onto type-IV collagen. Less than 5% of cells exhibited a flattened morphology. In contrast, more than 90% of KiSS-I transfectants had spread significantly in the same time.

KiSS-I expression also correlated with a significantly reduced ability to form colonies in both soft (0.3%) or hard (0.9%) agar (Fig. 4). This parameter was examined because of published reports demonstrating a correlation between clonogenicity on hard and/or soft agar and metastatic propensity (10).
BREAST CANCER METASTASIS SUPPRESSION

KiSS-I expression in melanoma does not correlate with decreased invasiveness, nor does it correlate with the ability to adhere to basement membrane components. These conclusions were corroborated in the MDA-MB-435 breast carcinoma model. The ultimate levels of adhesion to extracellular matrix components did not change; however, KiSS-I transfectants did spread on type-IV collagen more quickly than did control cells. The mechanism underlying this change is not known, but this is the first evidence that KiSS-I may be involved, at least tangentially, in cellular cytoskeletal organization.

Previous reports suggested that clonogenicity in agar, especially in hard agar, correlated with the metastatic potential of MDA-MB-435 cells (10). Our results generally agree with their findings. KiSS-I transfectants formed significantly fewer colonies than did control cells. Again, this finding is consistent with altered cell structure in KiSS-I transfectants.

In summary, transfection of the human melanoma metastasis suppressor gene KiSS-I into MDA-MB-435 human breast carcinoma cells significantly suppressed metastasis in athymic nude mice. This extends to three the number of cell lines transfected with KiSS-I that showed marked suppression of metastasis without alteration of tumor-forming ability. The results presented in this paper show that KiSS-I probably functions to control metastasis in a pathway shared by at least two tumor cell types of distinct embryonic origin, breast cancer and melanoma. Our results also imply that KiSS-I might be a gene mapping to chromosome 1q, which was previously implicated in breast cancer progression by loss-of-heterozygosity studies, and although they do not prove a role for KiSS-I in cytoskeletal organization, differential spreading on type-IV collagen and anchorage-independent growth are consistent with the predicted function of KiSS-I as an SH3 ligand.

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