Overexpression of ADAM9 in Non-Small Cell Lung Cancer Correlates with Brain Metastasis

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ABSTRACT

The “a disintegrin and metalloprotease” (ADAM) family contributes to regulation of the cell-cell and cell-matrix interactions that are critical determinants of malignancy. To determine the relationship between metastasis and ADAM proteins, we compared the mRNA levels of ADAM9, -10, -12, -15, and -17 in sublines of an EBC-1 lung cancer cell line that were highly metastatic to either brain or bone. ADAM9 mRNA levels were significantly higher in highly brain-metastatic sublines than in the parent or highly bone-metastatic sublines. To elucidate the role of ADAM9 in brain metastasis, we stably transfected A549 and EBC-1 cells with a full-length ADAM9 expression vector. Compared with mock-transfected, ADAM9 overexpression resulted in increased invasive capacity in response to nerve growth factor, increased adhesion to brain tissue, and increased expression of integrin a2 and p1 subunits. Administration of the anti-p1 monoclonal antibody attenuated this increase in invasive and adhesive activity. Intravenous administration of ADAM9-overexpressing A549 cells to mice resulted in micrometastatic foci in the brain and multiple metastatic colonies in the lungs. In contrast, administration of parent and mock-transfected A549 cells to mice resulted in lung tumors without brain metastasis. These results suggest that ADAM9 overexpression enhances cell adhesion and invasion of non-small cell lung cancer cells via modulation of other adhesion molecules and changes in sensitivity to growth factors, thereby promoting metastatic capacity to the brain.

INTRODUCTION

Surgical resection of primary non-small cell lung cancer is frequently followed by tumor recurrence at distant sites as a result of occult micrometastasis (1). In fact, despite improvements in the diagnosis and treatment of non-small cell lung cancer, the overall survival rate for patients with this disease has remained generally unchanged over the past decade, and most deaths are associated with metastasis to the brain, lungs, and bones (2).

Metastasis involves a series of events involving tumor cells, including release from the primary tumor, invasion of the surrounding pulmonary parenchyma, and movement into the lymphatic system or vasculature. Those cells that survive within the circulation migrate into parenchymal tissue at distant sites and establish metastatic growth (3). Invasion into these distant sites requires degradation of extracellular matrix (ECM) components (collagen IV, laminin, and fibronectin), which may be mediated by matrix metalloproteinases (4). Furthermore, cancer cell adhesion is also an essential cellular function in this process (5). Integrins are adhesion molecules that are involved in cell-matrix and cell-cell interactions and play a role in the development of tumor metastasis (6, 7). Aberrant integrin expression likely contributes to tumor growth and metastasis (8, 9).

The recently discovered ADAM family of proteins, so named because they contain a disintegrin and metalloprotease domain, is unique in that the members have the potential to regulate both ECM remodeling and cell migration (10, 11). Some ADAM proteins interact with integrins and thus may also play a role in metastasis of cancer cells (12). In fact, expression of some ADAM proteins is increased in malignant cell populations (13, 14), including cells obtained from pancreatic and hepatocellular carcinomas (15, 16) and from breast cancers (17). However, the precise role of ADAM proteins in malignancy remains unclear (18).

Thus, the goal of the present study was to characterize expression of various members of the ADAM family in cancer cell lines with different metastasis profiles, using in vitro and in vivo methods.

MATERIALS AND METHODS

Cell Culture and Reagents. A549 and EBC-1 cells were obtained from the Japanese Cancer Research Resources Bank and cultured in tissue culture flasks (Nalge Nunc, Naperville, IL) in DMEM (Nihonseiyaku, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Dainippon Pharmaceutical Co., Tokyo, Japan), 50 units/ml penicillin, and 50 µg/ml streptomycin (Life Technologies, Inc., Frederick, MD). A549 and EBC-1 cells were transfected with ADAM9 cDNA and maintained in DMEM containing 10% FBS and hygromycin (Wako, Osaka, Japan) in concentrations of 600 and 200 µg/ml, respectively.

In vivo selection was carried out to establish a highly metastatic subline of EBC-1, using the procedures described by Fidler (19). In brief, 1.0 × 106 of the parental EBC-1 cells in 100 µl of serum-free DMEM were injected into the left ventricles of 4-week-old female nude mice. Brain or bone marrow tissues containing the metastatic tumor cells were then excised, minced, and reimplanted into the left ventricle of a new recipient mouse for the selection of highly metastatic tumor cells. After five rounds of in vivo selection using sequential implantation, metastatic nodules in the tissues were harvested for in vitro culture of metastatic tumor cells.

Quantitation of ADAM mRNA Levels. Total RNA was extracted, by use of TRIZOL reagent (Life Technologies, Inc., Rockville, IN), from EBC-1 cells plated in 6-cm dishes. Genomic DNA contamination was eliminated by use of DNase (Promega, Madison, WI). Reverse transcription was carried out with 5 µg of total RNA, 0.5 µg of oligo(dT)12, 5 µl of 5× buffer, 2 µl of 2 mmol/L deoxynucleotide triphosphate mixture, 40 units of RNasin, and 5 units of avian myeloblastosis virus reverse transcriptase (Promega). The resulting mixture was incubated at 42°C for 60 min and then at 65°C for 15 min. PCR was carried out in a capillary reaction tube using a reaction mixture containing TaqMan Universal PCR Master Mix (PE Applied Biosystems, Foster City, CA), primer mixture, a labeled probe, and 2 µl of sample cDNA. The concentrations for primer mixtures and probes were 300 nm of each sense and antisense primer and 200 nm of each probe for ADAM mRNA, and 200 nm of each primer and 100 nm of probe for glyceraldehyde-3-phosphate dehydrogenase mRNA (Table 1). Real-time PCR was run on an ABI PRISM 5700 Sequence Detection System using the following PCR protocol: 50°C for 2 min and 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. After determination of the threshold cycle, which was defined as the PCR cycle number at which point the fluorescence intensity exceeded the threshold, standard curves for the quantitation of ADAM and glyceraldehyde-3-phosphate dehydrogenase were constructed. This was based on the relationship between the threshold cycle and the results of simultaneous amplification of serial dilutions of the cDNA from parent EBC-1 cells, whose ADAM mRNA level was defined as 1.0.
ADAM9 Forward primer TGGAACTGACAGAATGGCA
Reverse primer CCAAACAATGATTTTCTCCG
Probe TTGTTGGAAGCAGTGTTCTG

ADAM10 Forward primer ACGCCTTACGACACCAC
Reverse primer GGCTCTCTGCAGCGCTG
Probe AAGCGGACGACGGCA

ADAM12 Forward primer CTCTCGGGCGCCGGACGC
Reverse primer CAACTGCCAACCTGTACAA
Probe TTCTACCCTGGCCGGAGCC

ADAM15 Forward primer CAGCCTGCACCTGGAA
Reverse primer GCCAACATCGGCAAAAT
Probe CCAAAATAGCAGCACAGCTGCCAAG

ADAM17 Forward primer GGTTTGGAGAAAGCCCT
Reverse primer GTACCCTGCAGCTCAAAATG
Probe AAGGCTGGATGGCGTTCG

GAPDH Forward primer GAAGGTGAAGGTCGGAGTC
Reverse primer GAAGATGGTGATGGGATTTC
Probe GTGGGAACAGTGTGTTCAAGGAGCC

Table 1  Sequence of PCR primers and sequence-specific probes for members of the α-disintegrin-and-metalloprotease (ADAM) family and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

<table>
<thead>
<tr>
<th>Target</th>
<th>PCR primers and probes</th>
<th>Sequence (5 ’-3 ’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM9</td>
<td>Forward primer</td>
<td>TGGAACTGACAGAATGGCA</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>CCAAACAATGATTTTCTCCG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TTGTTGGAAGCAGTGTTCTG</td>
<td></td>
</tr>
<tr>
<td>ADAM10</td>
<td>Forward primer</td>
<td>ACGCCTTACGACACCAC</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>GGCTCTCTGCAGCGCTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>AAGCGGACGACGGCA</td>
<td></td>
</tr>
<tr>
<td>ADAM12</td>
<td>Forward primer</td>
<td>CTCTCGGGCGCCGGACGC</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>CAACTGCCAACCTGTACAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>TTCTACCCTGGCCGGAGCC</td>
<td></td>
</tr>
<tr>
<td>ADAM15</td>
<td>Forward primer</td>
<td>CAGCCTGCACCTGGAA</td>
<td>62</td>
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<td>Reverse primer</td>
<td>GCCAACATCGGCAAAAT</td>
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</tr>
<tr>
<td></td>
<td>Probe</td>
<td>CCAAAATAGCAGCACAGCTGCCAAG</td>
<td></td>
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<tr>
<td>ADAM17</td>
<td>Forward primer</td>
<td>GGTTTGGAGAAAGCCCT</td>
<td>226</td>
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<td></td>
<td>Reverse primer</td>
<td>GTACCCTGCAGCTCAAAATG</td>
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<tr>
<td></td>
<td>Probe</td>
<td>AAGGCTGGATGGCGTTCG</td>
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The amount of ADAM mRNA in the sample was calculated from the standard curves. To normalize the difference in RNA degradation and RNA loading for RT-PCR in individual samples, we divided the levels of ADAM mRNA by those of glyceraldehyde-3-phosphate dehydrogenase mRNA and defined as relative ADAM expression. Quantitation of ADAM mRNA levels in EBC-1 cells was performed in duplicate and repeated three times.

Western Blot Analysis. Cells were grown in 6-cm dishes, washed with PBS, and lysed directly on the dish with lysis buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and protease inhibitors on ice for 30 min. The lysates were clarified by centrifugation at 14,000 rpm for 10 min and were mixed with loading sample buffer, heated 5 min at 95°C, separated by SDS-PAGE, and transferred to nitrocellulose. The nitrocellulose filters were blocked with 5% reconstituted dry milk overnight at 4°C, washed with PBS, and lysed directly on the dish with lysis buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and protease inhibitors on ice for 30 min. The lysates were clarified by centrifugation at 14,000 rpm for 10 min and were mixed with loading sample buffer, heated 5 min at 95°C, separated by SDS-PAGE, and transferred to nitrocellulose.

Flow Cytometry Analysis. To stain integrins on the cell surface, cells were harvested, rinsed, and suspended in DMEM (supplemented with 1% FBS and 0.03% sodium azide), and sequentially incubated for 30 min at 4°C with mAbs (10 μg/ml) against the human integrin β1 (Becton Dickinson Bioscience, Bedford, MA), α1 (Upstate Biotechnology, Lake Placid, NY), α2 (Chemicon), α3 (Becton Dickinson Bioscience), and α6 (Immunotech, Marseille, France) subunits. After washing, the cells were incubated with FITC-conjugated mouse IgG (DAKO, Copenhagen, Denmark) for 30 min at 4°C. Absence of the primary antibody served as a negative control in each case. The cells were washed, resuspended, and analyzed by use of a FACS Calibur (Becton Dickinson Biosciences, Oxford, CA).

Statistical Analysis. In vitro invasion assays and in situ adhesion assays were performed in duplicate and repeated three times with three clones of each cell line. Flow cytometry analysis was performed with three clones of each cell line. Mann–Whitney U tests and Kruskal–Wallis tests were performed for comparison of the results (Starview version 5.0 for Windows; Abacus Concepts, Berkeley, CA). P < 0.05 was considered to indicate statistical significance.

RESULTS

ADAM mRNA Levels in EBC-1 Cell Lines. ADAM mRNA levels in the EBC-1 cell lines are shown in Fig. 1A. ADAM9 mRNA level were significantly higher in the highly brain-metastatic EBC-1 cells than in the parent or the highly bone-metastatic EBC-1 cells.

ADAM9 and ADAM17 Protein Expression in EBC-1 Cell Lines. ADAM9 and ADAM17 protein expression levels in the EBC-1 cell lines are shown in Fig. 1B. Whereas there was no significant difference in ADAM17 expression in EBC-1 cell lines, ADAM9 protein expression was higher in the highly brain-metastatic EBC-1 cells than in the other cell lines.
Inducible Overexpression of ADAM9 in A549 and EBC-1 Cells.

To determine the role of ADAM9 in metastatic potential, overexpression of ADAM9 was induced in A549 and EBC-1 cells (ADAM9-A549 and ADAM9-EBC-1 cells). Cell-surface biotinylation and immunoprecipitation experiments demonstrated the presence of ADAM9 (an 84-kDa fragment in a reduced condition) on the cell surface (Fig. 2). Three clones overexpressing ADAM9 were selected from each cell line and used for subsequent experiments. Overexpression of ADAM9 had no effect on cell morphology or on proliferation rate (data not shown).

Overexpression of ADAM9 Alters NGF-Induced Cell Migration and Invasion.

To investigate the role of neurotrophic factors in cell motility, we performed Matrigel assays, using NGF as a chemoattractant. These experiments demonstrated that NGF-induced invasion was significantly greater when ADAM9 transfectants were used compared with mock-transfected cells (Fig. 3).

Integrin Expression in Transfectant Cells. To further characterize the relationship between ADAM9 and invasive and adhesive increased adhesion to brain tissues, we performed a cell–brain tissue adhesion assay. Adhesion to brain tissue was significantly higher in ADAM9-A549 and ADAM9-EBC-1 cells than in mock-transfected cells (Fig. 4).

In Situ Adhesion Assay of Frozen Brain Tissues.

To investigate whether ADAM9-overexpressing lung cancer cells exhibited increased adhesion to brain tissues, we performed a cell–brain tissue adhesion assay. Adhesion to brain tissue was significantly higher in ADAM9-A549 and ADAM9-EBC-1 cells than in mock-transfected cells (Fig. 4).
capacity in brain tissues, we assessed expression of integrins. Cell-surface expression of integrin α3 and β1 subunits was higher in ADAM9-A549 and ADAM9-EBC-1 cells than in mock-transfected cells (Fig. 5). However, expression of the α1, α2, and α6 subunits was similar among all cell lines. Preincubation of cells with the integrin-β1-blocking mAb resulted in an attenuation of invasive activity (Fig. 6A) and adhesion to brain tissues (Fig. 6, B and C).

Overexpression of ADAM9 Induces Brain Metastasis: *In Vivo* Metastasis Assay. Twelve weeks after i.v. administration of parent A549, mock-transfected A549, and ADAM9-transfected A549 cells, multiple tumor foci were seen in the lung tissues of all animals (Table 2; Fig. 7, A and B). There were no differences in the number of metastatic colonies in the lungs of animals injected with parent A549 cells, mock-transfected, or ADAM9-transfected. Although no tumors were observed in other organs in animals that received parent A549 cells (n = 5) or mock-A549 cells (n = 5), H&E staining and CEA immunostaining demonstrated the presence of microscopic metastasis in the brains of 15 of 18 mice receiving injections of ADAM9-A549 cells (Fig. 7, C–F). Because CEA is not expressed in mouse cells, positive CEA immunostaining in these cases suggests that the microscopic foci are composed of human cancer cells. Some mice receiving injections of ADAM9-A549 cells demonstrated neurological symptoms (e.g., paralysis), which is consistent with the presence of brain metastasis.

**DISCUSSION**

ADAM9 is a widely expressed, catalytically active metalloprotease–disintegrin protein that is highly conserved in humans and mice (22). The present study demonstrates that ADAM9 mRNA levels are significantly higher in the highly brain-metastatic EBC-1 subline and that ADAM9 overexpression in A549 and EBC-1 cells resulted in an increase in NGF-induced invasive capacity and cell adhesion to brain tissue. Furthermore, i.v. injection of ADAM9-overexpressing A549 cells into nude mice resulted in brain metastasis. Together, these data suggest that increased expression of ADAM9 correlates with invasive and adhesive potential and that ADAM9 participates in the induction of tumor cell trafficking to the brain. This is the first study to...
demonstrate a relationship between cancer metastasis and ADAM9 expression.

Members of the ADAM family, including ADAM9, -10, -12, -15, and -17, contain the amino acid sequence HEXXHXXGXXH in the catalytic zinc-binding site, which confers the functional metalloprotease capacity. Specifically, the recombinant metalloprotease domain of ADAM9 shows activity against gelatin, β-casein, and fibronectin (23). Therefore, it is likely that this metalloprotease domain plays a role in the ECM degradation or basement membrane modifications required for successful metastasis (13). Indeed, in the present study, the increased invasive capability of ADAM9-A549 and ADAM9-EBC-1 cells was demonstrated by Matrigel assay. ADAM9 may either directly degrade the ECM or induce activation of other ECM proteases, such as matrix metalloproteinases, thereby allowing tumor cell penetration into the matrix of the brain.

Increased expression of integrin β1 also results in increased cell invasion and metastasis in various tumor types, including malignant melanoma, malignant glioma, colorectal cancer, and breast cancer (24–27). Integrin α3β1 is associated with gelatinase B (matrix metalloproteinase-9) activity (28). In the present study, integrin α3β1 expression was up-regulated in ADAM9-overexpressing cells, and preincubation of cells with the integrin-β1-blocking mAb resulted in a decrease in invasive activity. These results suggest that up-regulation of integrin α3β1 may play an important role in the invasive potential of ADAM9-overexpressing cells.

A plasma membrane-anchored disintegrin domain may play a role in cell–cell interactions by functioning as an integrin ligand (29, 30). Recent studies reported that ADAM9 and integrin β1 colocalize (31) and that ADAM9 regulates the motility of cells by binding to integrin αβ1 (32, 33). These integrin-regulating functions make ADAM9 an ideal candidate to serve as an adhesion- and migration-regulating molecule (34). Taken together, these data suggest that ADAM9 regulates tumor adhesion and invasion to brain tissues through modulation of integrin function in cancer cells.

Metastasis to the brain requires that malignant cells undergo a series of events, including attachment to the endothelial cells of the brain microvessels, activation by brain endothelial cell-derived motility factors and brain-derived invasion factors, invasion through the blood–brain barrier, and activation by brain-derived survival and growth factors (35). Paget (36) suggested that certain tumor cells (seeds) have a specific affinity for the milieu of certain organs (soil) and that metastasis occurs only when the seeds and soil are compatible. This implies that metastasis depends on interactions between tumor cells and the organ microenvironment. In the present study, ADAM9-A549 cells survived, invaded, and proliferated within the brain, indicating that they are compatible with the local brain microenvironment.

The proliferation of human lung cancer is regulated by several growth factors, both in vivo and in vitro, via an autocrine or paracrine mechanism (37). NGF enhances cancer cell growth and invasion in malignant melanoma, pancreatic cancer, prostate cancer, and lung cancer cells (38–41), whereas TrkA, a NGF high-affinity receptor recognized by a tyrosine-specific protein kinase, may modulate the biological activity of NGF in lung neoplasm (37). In the present study, ADAM9 transfectants showed an increase in NGF-induced invasion compared with mock-transfected cells. These data suggest that NGF functions as a chemoattractant to ADAM9-transfected cells and promotes tumor cell penetration into the matrix of the neurotrophin-rich stromal brain microenvironment.

In conclusion, ADAM9 overexpression enhanced cancer cell adhesion and invasion via modulation of other adhesion molecules and changes in sensitivity to growth factors. Furthermore, overexpression of ADAM9 promoted cancer cell trafficking to the brain.

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**Table 2: Metastasis of A549 wild-type, mock-transfected A549, and α-disintegrin- and-metalloprotease-9 (ADAM9)-transfected A549 cells after i.v. administration to nude mouse**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cells</th>
<th>Mice used (n)</th>
<th>Cases with lung metastasis (n)</th>
<th>Cases with brain metastasis (n)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Wild-type A549</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Mock-transfected A549</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>ADAM9-transfected A549 clone 1</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>ADAM9-transfected A549 clone 2</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>ADAM9-transfected A549 clone 3</td>
<td>6</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

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**Fig. 6.** A, suppression of invasive activity by inhibition of integrin β1. Invasive activity was determined as the numbers of cells that invaded to the lower side through the filter pores as described in “Materials and Methods.” The columns represent mean values of independent experiments measured in duplicate and repeated three times using three clones of each cell line, and the bars represent the SD ([+], with anti-integrin-β1 monoclonal antibody (mAb); [–], without anti-integrin-β1 mAb). The nerve growth factor-induced invasive activity of a disintegrin and metalloprotease 9 (ADAM9)-overexpressing cells was reduced to control levels after treatment with the integrin-β1-blocking mAb (+, $P < 0.05$, Mann–Whitney U test). B, suppression of in situ adhesion assay by inhibition of integrin β1. Confocal laser microscopy of EBC-1 cells labeled with CellTracker green 5-chloromethylfluorescein diacetate on the brain tissues. Mock- and ADAM9-transfected cells are stained green, and the nuclei of brain cells are stained red by propidium iodide, as described in “Materials and Methods.” C, adhesive activity was determined as the number of cells that adhered to the frozen tissues as described in “Materials and Methods.” The columns represent mean values of independent experiments measured in duplicate and repeated three times using three clones of each cell line, and the bars represent the SD. Preincubation of the cells with the integrin-β1-blocking mAb led to a significant decrease in adhesion activity (*, $P < 0.05$, Mann–Whitney U test). HPF, high-power field.
Fig. 7. In vivo metastasis assay. Five or six animals in each group of nude mice received an i.v. injection of $10^6$ cells. After 3 months, the mice were euthanized, and their organs were removed and examined. A, mice receiving A549 cells developed multiple colonies in the lungs (arrows). B, microscopic appearance of metastatic foci in the lungs of the mouse receiving A549 cells. (H&E staining; ×40 magnification). C and D, microscopic appearance of micrometastatic foci in the brain of the mice receiving a disintegrin and metalloprotease 9 (ADAM9)-A549 cells. Mice receiving ADAM9-overexpressing A549 cells developed multiple micrometastatic foci (arrow in C) in the cerebral cortex (H&E staining; magnification, C, ×40; D, ×100). E, immunoperoxidase staining shows carcinomaembryonic antigen localization in the metastatic lesion exclusively (magnification, ×100). F, micrometastatic cancer cells near the brain blood vessel (H&E staining; magnification, ×100).

ACKNOWLEDGMENTS

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