EphA2 Overexpression Causes Tumorigenesis of Mammary Epithelial Cells

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ABSTRACT

Elevated levels of protein tyrosine phosphorylation contribute to a malignant phenotype, although the tyrosine kinases that are responsible for this signaling remain largely unknown. Here we report increased levels of the EphA2 (ECK) protein tyrosine kinase in clinical specimens and cell models of breast cancer. We also show that EphA2 overexpression is sufficient to confer malignant transformation and tumorigenic potential on nontransformed MCF-10A mammary epithelial cells. The transforming capacity of EphA2 is related to the failure of EphA2 to interact with its cell-associated ligands. Interestingly, stimulation of EphA2 reverses the malignant growth and invasiveness of EphA2-transformed cells. Taken together, these results identify EphA2 as a powerful oncoprotein in breast cancer.

INTRODUCTION

Cancer is a disease of aberrant signal transduction. In the search for signals that cause breast cancer, many lines of investigation have linked cancer with elevated expression or altered function of receptor tyrosine kinases (1). Recent studies have identified overexpression of HER2 or epidermal growth factor receptor in some tumors and used this knowledge to develop successful approaches for therapeutic targeting of cancer cells (2). However, overexpression of HER2 and epidermal growth factor receptor is limited to a subset of tumors, which creates a need to identify other tyrosine kinases that are responsible for cancer progression and pathogenesis.

When malignant cells metastasize to distant sites in the body, morbidity and mortality increase significantly (3). Metastatic cells have acquired the abilities to break away from the primary tumor, translocate to distant sites in the body, and colonize a foreign microenvironment (4). At the cellular level, malignant cells have overcome restraints on cell growth and migration that result from physical linkages and signals conveyed by cell-cell contacts (5). Malignant cells often have increased interactions with surrounding ECM proteins, which provide linkages and signals that promote several aspects of metastasis (6).

Our previous studies revealed that the levels of protein tyrosine phosphorylation regulate a balance between cell-cell and cell-ECM adhesions in epithelial cells (7). Using oncogene-transformed mammary epithelial cells, we showed that elevated tyrosine kinase activity weakens cell-cell contacts and promotes ECM adhesions (7). To identify tyrosine kinases that control tumor cell adhesion, we developed novel technologies to generate monoclonal antibodies against tyrosine kinases in cancer cells (8). We focused on one particular antigen that was functionally altered in oncogene-transformed epithelial cells. This antigen was identified as EphA2 (ECK). EphA2 is a 130,000 receptor tyrosine kinase that is expressed on adult epithelia (9), where it is found at low levels and enriched within sites of cell-cell adhesion (10). The subcellular localization is important because EphA2 binds five different ligands, ephrinA1–5, which are attached to the cell membrane (11).

MATERIALS AND METHODS

Cells and Antibodies. All cells were cultured as described previously (10). Monoclonal antibodies against EphA2 were generated in our laboratory (D7 and B2D6; Ref. 8) or purchased from Upstate Biologicals, Inc. (Lake Placid, NY). Antibodies specific for β-catenin and P-Tyr (PY-20) were purchased from Transduction Laboratories (Lexington, KY). Antibodies specific for P-Tyr (4G10) were purchased from Upstate Biologicals, Inc. EA1 was a generous gift from Dr. B. Wang (Case Western Reserve University, Cleveland, OH).

Western Blot Analysis and Immunoprecipitation. Western blot analyses were performed as described previously (10), and antibody binding was detected by enhanced chemiluminescence (Pierce, Rockford, IL) and autoradiography (Kodak X-OMAT; Kodak, Rochester, NY). To confirm equal sample loading, the blots were stripped and reprobed with antibodies specific for β-catenin or vinculin.

Immunohistochemistry and Immunofluorescence Staining. Formalin-fixed, paraffin-embedded “sawage” slides, each containing 15–20 breast cancer specimens (kindly provided by B. J. Korns; BioGenex, San Ramon, CA), were stained and scored as described previously (12). Mean immunostaining intensity in benign and malignant breast was compared using Student’s t test with statistical software (SAS for Windows v.6.04 and Microsoft Excel ‘97), defining P < 0.05 as significant. Staining of cell monolayers with EphA2 antibodies (clones D7 or B2D6) was performed as described previously (10).

Transfection and Selection. Monolayers of MCF-10A cells were cotransfected with the pNeoMSV-EphA2 (generously provided by Dr. T. Hunter, Scripps Institute, La Jolla, CA) and pBabe-Puro eukaryotic expression vectors, at a 4:1 ratio, using LipofectAMINE Plus (Life Technologies, Inc., Grand Island, NY). As a control for the transfection procedure, a parallel transfection was performed using pNeoMSV and pBabe-Puro. Puromycin-resistant cells were selected by supplementing the growth medium with 1 μg/ml puromycin (Sigma, St. Louis, MO). EphA2 overexpression was confirmed by Western blot analysis with specific antibodies. All experiments were performed using bulk culture transfecants, and identical results were obtained using cells from two separate transfections with EphA2 cDNAs. Parental cells and cultures transfected with pBabe-Puro were used as negative controls.

Colony Formation in Soft Agar. Colony formation in soft agar was performed as described previously (13). Colony formation was scored microscopically, and clusters of at least three cells were defined as a positive result. For experiments with EA1, 0.5 μg/ml EA1 or a matched vehicle (50% glycerol in PBS) was included in top agar solution, and ligand was replenished daily with fresh media. The data shown are pooled from 10 separate high-power microscopic fields from each sample and representative of at least three separate experiments.

Cell Behavior in Matrigel. The behavior of cells in Matrigel was analyzed as described previously (14). Briefly, tissue culture dishes were coated with Matrigel (Collaborative, Bedford, MA) at 37°C before adding 1 × 10⁶ vector- or EphA2-transfected MCF-10A cells. The behavior of EphA2-overexpressing cells was assessed at 6-h intervals using an inverted light microscope (Olympus IX-70). For experiments with EA1, the culture medium was supplemented with 0.5 μg/ml EA1 or an appropriately matched vehicle control. All images were recorded onto 35-mm film (T-Max-400; Kodak, Rochester, NY).

 Xenograft Analyses. Athymic (nu/nu) 3–4-week-old mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and Charles River (Wilmington, MA) and acclimated for 7–10 days. For s.c. implantation, 1 × 10⁶ or 5 × 10⁶ vector- or EphA2-transfected MCF-10A cells were suspended in 100 μl of PBS and subcutaneously injected into the right flank of each mouse. Mice were killed 7–12 weeks after s.c. implantation, and tumors were removed, weighed, and measured. Data are expressed as mean tumor volume ± SE. The experiments were repeated three times with comparable results. Significance was determined using Student’s t test.
µl of fresh media and injected into the right craniolateral thorax (axilla) using a 23-gauge needle. For tail vein injections, 1 x 10^6 cells were injected into the tail vein, and mice were monitored for 7–28 days. At necropsy, primary tumors and all organs were evaluated macroscopically for the presence of tumors. Tissue samples of the primary tumor and organs were fixed in 10% buffered neutral formalin and embedded in paraffin. Tissue sections of the tumors and lung were stained with H&E to assess morphology. Lung sections were stained with antibodies specific for cytokeratin (AE1/AE3) or factor VIII-related antigen (DAKO, Carpinteria, CA) to confirm the epithelial nature of lung metastases.

RESULTS

Elevated EphA2 Protein Levels in Breast Cancer Cells. The levels of EphA2 protein were measured in clinical specimens of benign or malignant mammary glands (Fig. 1A). Immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections revealed a low level of EphA2 immunoreactivity in benign mammary epithelia, with an average staining intensity of 0.1 (using a 0–3 scale to report staining intensity; Fig. 1B). EphA2 immunoreactivity was increased in breast carcinoma specimens, with an average staining intensity of 2.9. Interestingly, EphA2 immunoreactivity in the breast carcinoma cells was diffusely distributed throughout the cytoplasm (Fig. 1A, top). Increased staining intensity was accompanied by a larger percentage of carcinoma cells (an average of 87%) that stained positive for EphA2 as compared with benign mammary epithelial cells (an average of 3%).

The elevated levels of EphA2 in clinical specimens prompted us to measure EphA2 in cell models of nontransformed breast epithelia (MCF-10A, MCF-12A, and MCF-10-2) and aggressive breast cancer epithelia [Hs578T, MDA-436, MDA-435, MDA-231, and BT549 (15, 16)]. Equal amounts of whole cell extracts were resolved by SDS-PAGE and subjected to Western blot analysis using EphA2-specific antibodies. Whereas lower levels of EphA2 protein were detected in nontransformed epithelial cells (Fig. 2A, Lanes 1–3), more EphA2 was detected in aggressive carcinoma cells (Fig. 2A, Lanes 4–8). Identical results were obtained when equivalent numbers of cells or equal amounts of protein were analyzed (data not shown). Increased levels of EphA2 in aggressive cancer cell models were also confirmed using different EphA2 antibodies (D7, B2D6, and EK166B; data not shown), revealing that the differences in EphA2 levels did not reflect changes in a single epitope. The blots were stripped and reprobed with antibodies specific for β-catenin (Fig. 2A) or vinculin (data not shown), which confirmed equal sample loading.

EphA2 Overexpression in MCF-10A Cells. Because EphA2 was overexpressed in a large number of breast cancers, we assessed the consequences of EphA2 overexpression in nontransformed mammary epithelial cells. MCF-10A cells were transfected with human EphA2 cDNA (EphA2) or a vector control (Vector; Fig. 2B, top panel). After establishing cultures of MCF-10A cells with stable overexpression of EphA2, microscopic evaluation revealed differences in the cell morphology as compared with vector-transfected control cells (Fig. 2C). Nontransformed MCF-10A cells displayed an epithelial morphology and interacted with one another, even at low cell density. In contrast, EphA2-overexpressing MCF-10A cells (MCF-EphA2 cells) adopted a fibroblast-like morphology and did not form cell-cell contacts, even at high cell density (data not shown). To confirm that the mesenchymal morphology did not represent clonal variation, a separate sample of MCF-10A cells was transfected with EphA2 cDNAs and yielded identical results.

EphA2 Overexpression Decreases Ligand-mediated Stimulation. Because stable cell-cell contacts cause EphA2 to become enriched within sites of cell-cell contact (10), we assessed EphA2 subcellular localization by immunostaining with specific antibodies (Fig. 2C). The EphA2 on nontransformed MCF-10A cells was restricted to a narrow line where adjacent cells came into direct contact with each other, with little staining of membrane that was not in contact with neighboring cells. In contrast, the pattern of EphA2 staining on MCF-EphA2 cells was diffuse, with little staining of cell-cell contacts. Notably, the cytoplasmic immunoreactivity of EphA2, which was prominent in tumor specimens, was also observed in MCF-EphA2 cells.

The lack of EphA2 within the cell-cell contacts of MCF-EphA2 cells was intriguing because EphA2 is stimulated by ligands that are anchored to the cell membrane (11, 17). To measure EphA2 stimu-
EphA2 OVEREXPRESSION CAUSES TUMORIGENESIS

Table 1: Summary of EphA2-overexpressing MCF-10A cells

<table>
<thead>
<tr>
<th>Subcellular localization</th>
<th>P-Tyr content</th>
<th>Soft agar colonization</th>
<th>Behavior in 3DBM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector Cell-cell contacts</td>
<td>High</td>
<td>Negative</td>
<td>Benign</td>
</tr>
<tr>
<td>EphA2 Membrane ruffles</td>
<td>High</td>
<td>Low</td>
<td>Aggressive (stellate)</td>
</tr>
</tbody>
</table>
| EphA2 Overexpression Confers Tumorigenic Potential. Because in vitro analyses of transformation do not always predict tumorigenic potential in vivo, control or EphA2-overexpressing MCF-10A cells were implanted in athymic (nu/nu) mice (Fig. 4). The s.c. injection of MCFEphA2 cells caused the formation of palpable tumors within 4 days (Fig. 4, A–C) in 19 of 19 mice. The median volume of resulting tumors was related to the number of implanted cells and reached an average of 300 mm3 (for samples injected with 5 x 106 cells) within 10 days (Fig. 4E). Necropsy revealed that the tumors were firmly attached to the underlying axillary muscle and surrounded by fibrous tissue (data not shown). Histologically, the neoplastic cells were invasive and associated with fibrous connective tissue (Fig. 4, B and C). These neoplastic cells exhibited moderate cyttoplasmic and nuclear pleomorphism and formed dysplastic tubular and secreting structures. In control experiments, cells transfected with vector DNA failed to grow in athymic mice (0 of 13 mice; Fig. 4E), and necropsy failed to identify any growth or invasion of these cells (data not shown).

Because the highest levels of EphA2 were consistently found in breast cancer cells that are invasive in vivo (16), 1 x 106 control or MCFEphA2 cells were injected into the tail vein of athymic mice. Within 7 days, necropsy revealed lung emboli within large- and medium-sized vessels in two of four mice injected with MCFEphA2 cells. The emboli were generally found to occlude large blood vessels.
MCF EphA2 cells displayed a stellate growth pattern in Matrigel that mimicked the behavior of aggressive breast cancer cells (MDA-MB-231; data not shown). Note that treatment with 0.5 mM EA1 caused the phenotype of MCF EphA2 cells to be indistinguishable from that of control MCF-10A cells.

Fig. 3. EphA2 overexpression induces malignant transformation but is reversed by ligand binding. A, to measure anchorage-independent cell growth and survival, 1 x 10⁶ vector- or EphA2-transfected MCF-10A cells were suspended in soft agar in the presence or absence of 0.5 μg/ml EA1. After 7 days, colony formation was scored microscopically, and clusters containing at least 3 cells were defined as a positive colony. MCF EphA2 cells demonstrated significant increases in anchorage-independent growth (*, P < 4 x 10⁻⁴), whereas EA1 treatment significantly blocks the growth of MCF EphA2 cells (**, P < 5 x 10⁻⁶). B, the phenotype of control and EphA2-transformed MCF-10A cells was evaluated after incubation atop polymerized Matrigel ± 0.5 μg/ml EA1 (bottom panels) or an appropriately matched vehicle (PBS; top panels). Whereas control MCF-10A cells were organized into spherical colonies, MCF EphA2 cells displayed a stellate growth pattern in Matrigel that mimicked the behavior of aggressive breast cancer cells (MDA-MB-231; data not shown). Note that treatment with 0.5 μg/ml EA1 caused the phenotype of MCF EphA2 cells to be indistinguishable from that of control MCF-10A cells.

but did not breach the vessel wall (Fig. 4D). Immunohistochemical staining with cytokeratin antibodies confirmed the epithelial nature of the emboli, and a lack of anti-factor VIII-related antigen immunoreactivity revealed that the thrombus did not represent an abnormal or atypical outgrowth of endothelial cells (data not shown). No pulmonary emboli were observed in mice that had been injected with control MCF-10A cells (Fig. 4E).

DISCUSSION

The major finding of this study is that the EphA2 tyrosine kinase is overexpressed in many clinical specimens and cell models of breast cancer. We have also shown that EphA2 overexpression is sufficient to induce malignant transformation of nontransformed epithelial cells. Importantly, EphA2 overexpression destabilizes cell-cell contacts and thereby prevents EphA2 from interacting with its ligands, which are anchored to the membrane of adjacent cells. This defect in ligand-mediated stimulation of EphA2 is important because we show that ligand binding reverses the malignant phenotype of EphA2-overexpressing cells.

Consistent results with several cell models suggest that elevated levels of EphA2 are relevant to breast cancer. High levels of EphA2 are found in a large number of clinical specimens and aggressive cell models of breast cancer. Recent studies reveal that high levels of EphA2 may also be found in advanced melanoma (20), colon cancer (21), and prostate cancer (12). The fact that elevated EphA2 levels are found on multiple types of cancer suggests that EphA2 overexpression may be a common event in the metastatic progression of carcinoma cells.

Our results provide the first evidence that EphA2 is not merely a marker but an active participant in tumorigenesis. EphA2-overexpressing MCF-10A cells displayed the hallmarks of malignant transformation as defined in vitro and in vivo. EphA2-transformed MCF-10A cells formed tumors in vivo at a high frequency, which is remarkable given that other oncogenes (e.g., Ras, HER2, and TC21) are insufficient to convey tumorigenic potential on MCF-10A cells (13, 14). Thus, we suggest that EphA2 overexpression may be particularly relevant to breast cancer.

EphA2 overexpression causes defects in cell-cell contacts that are characteristic of aggressive cancer cells. EphA2 weakens cell-cell contacts and thereby prevents EphA2 from interacting with its ligands, which are anchored to the surface of neighboring cells. Consistent with this, the highest levels of EphA2 are consistently found on tumor-derived breast cell lines that have weak cell-cell contacts (10, 16). Moreover, the EphA2 in these aggressive cancer cells is not tyrosine phosphorylated (10). One possible explanation for the weakened cell-cell adhesions is that overexpressed EphA2 may phosphorylate adhesion or cytoskeletal proteins and thereby destabilize cell-cell adhesions. Consistent with this, elevated levels of protein tyrosine phosphorylation have been shown to destabilize cell-cell adhesions (7, 22). Further support is provided by evidence that EphA2 interacts with important adhesion and cytoskeletal proteins, including E-cadherin, Src-like adapter protein, and phosphatidylinositol 3'-kinase (10, 18, 23). Another possibility is that EphA2 alters the expression of important adhesion molecules. Future studies will be needed to identify the molecular targets of EphA2 in malignant cells.

The weakened cell-cell adhesions of EphA2-overexpressing cells are notable because EphA2 binds a membrane-anchored ligand. We have recently shown that EphA2 in nontransformed epithelia is enriched within sites of cell-cell contact, where it interacts with ligand and becomes tyrosine phosphorylated (10). We demonstrate here that EphA2 overexpression causes EphA2 to become diffusely distributed. Consequently, the overexpressed EphA2 fails to interact with ligand and becomes tyrosine phosphorylated. Interestingly, EphA2 immunoreactivity in EphA2-overexpressing cells and in clinical specimens of breast cancer was similarly diffuse and cytoplasmic (see Fig. 1A). The cytoplasmic localization of EphA2 contrasts with its known localization within sites of cell-cell contact between nontransformed epithelial cells (9). These results lead us to postulate that the levels of EphA2 protein influence its subcellular localization and thereby regulate ligand binding.

EphA2 overexpression causes malignant transformation and decreases ligand binding. These properties appear to be directly linked because EphA2 stimulation by soluble ligands reverses the malignant behavior of EphA2-transformed cells. Ligand-mediated tyrosine phosphorylation of EphA2 also decreases the growth and invasiveness of malignant breast and prostate cancer cells (10, 18). Thus, whereas ligand binding inhibits tumor cell growth, EphA2 overexpression causes malignant transformation and tumorigenesis. Taken together, these results indicate that the expression levels and ligand binding
properties work together to allow EphA2 to differentially regulate tumor cell growth and invasiveness.

EphA2 overexpression may cause malignant transformation by regulating cell contact with the ECM. In contrast to evidence that ligand-mediated stimulation of EphA2 blocks ECM attachments (10, 18), ECM adhesions are increased in EphA2-transformed MCF-10A cells relative to nontransformed epithelial cells (4). Many different lines of investigation have shown that ECM adhesions provide linkages and signals that promote cell growth, migration, and survival (6, 24, 25). The molecular basis by which EphA2 regulates ECM adhesions remains largely unknown. However, EphA2 has been shown to interact with a variety of cytoskeletal and signaling proteins, including phosphatidylinositol 3'-kinase, FAK, SHP-2, and a Src-like adapter protein (18, 23, 26). These protein interactions are intriguing because each of the associated proteins has been independently found to regulate cell growth or ECM adhesion (24, 27, 28).

Overexpressed receptor tyrosine kinases can facilitate new and efficacious modalities for targeted intervention against cancer cells (2). A recent success arose from antibody targeting of HER2, a receptor tyrosine kinase that is overexpressed on some breast cancer cells (2). Unfortunately, HER2 overexpression is limited to one-third of breast carcinomas and is sporadic on other tumor types, which underscores the need for new targets. Our results suggest that EphA2 might provide a target for intervention against aggressive breast cancers. At minimum, EphA2 overexpression may identify a larger or different set of tumors than HER2. Strong EphA2 immunoreactivity was detected in 5 of 12 (40%) breast cancer specimens, whereas strong HER2 immunoreactivity was limited to 2 of 12 samples (data not shown). Our evidence suggests that strategies that restore or mimic the effects of ligand could negatively regulate tumor cell

Fig. 4. EphA2 overexpression conveys tumorigenic potential. A–C, MCF\textsuperscript{EphA2} cells were implanted s.c. into the right craniolateral thorax (axilla) of athymic (nu/nu) mice. A, within 4 days, the implanted cells formed palpable masses (arrowheads). B, the histological appearance of the tumor revealed that these masses were almost entirely composed of moderately differentiated and invasive tumor cells (arrowheads) that formed dysplastic tubules with fluid-filled lumens (L). C, neoplastic cells (arrowheads) invaded adjacent skeletal muscle fibers (m). D, MCF\textsuperscript{EphA2} cells injected i.v. into the tail vein of athymic mice formed emboli in the lung within large- and medium-sized vessels (arrowheads). Histological examination of pulmonary tumor thrombi in athymic mice revealed that tumor cells partially to totally obstructed intravascular spaces but did not invade the vessel wall. Bar, 40 μm.

E, tumorigenesis by EphA2-transformed MCF-10A cells (or vector-transfected controls) was evaluated after s.c. or tail vein injection. The significance of tumor formation was estimated to be $P < 1.3 \times 10^{-7}$ as determined by $\chi^2$ analyses.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Site of Inoculation</th>
<th># of Cells Injected</th>
<th>Incidence of Tumorigenicity</th>
<th>Tumor Volume (mm(^3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Subcutaneous</td>
<td>$1 \times 10^4$</td>
<td>0/9</td>
<td>NA</td>
</tr>
<tr>
<td>EphA2</td>
<td>Subcutaneous</td>
<td>$1 \times 10^4$</td>
<td>9/9</td>
<td>66 +/- 20</td>
</tr>
<tr>
<td>Control</td>
<td>Subcutaneous</td>
<td>$5 \times 10^4$</td>
<td>0/4</td>
<td>NA</td>
</tr>
<tr>
<td>EphA2</td>
<td>Subcutaneous</td>
<td>$5 \times 10^4$</td>
<td>10/10</td>
<td>293 +/- 70</td>
</tr>
<tr>
<td>Control</td>
<td>Tail vein</td>
<td>$1 \times 10^5$</td>
<td>0/4</td>
<td>NA</td>
</tr>
<tr>
<td>EphA2</td>
<td>Tail vein</td>
<td>$1 \times 10^5$</td>
<td>2/4</td>
<td>NA</td>
</tr>
</tbody>
</table>

\(^4\) D. P. Zelinski and M. S. Kinch, unpublished results.
growth and invasiveness (10, 18). This latter approach would redirect
the function of an overexpressed oncoprotein so that it blocks tumor
cell growth and invasiveness.

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