Differential EphA2 Epitope Display on Normal versus Malignant Cells

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ABSTRACT

The EphA2 receptor tyrosine kinase is overexpressed in many different types of human cancers where it functions as a powerful oncoprotein. Dramatic changes in the subcellular localization and function of EphA2 have also been linked with cancer, and in particular, unstable cancer cell-cell contacts prevent EphA2 from stably binding its ligand on the surface of adjoining cells. This change is important in light of evidence that ligand binding causes EphA2 to transmit signals that negatively regulate tumor cell growth and invasiveness and also induce EphA2 degradation. On the basis of these properties, we have begun to target EphA2 on tumor cells using agonistic antibodies, which mimic the consequences of ligand binding. In our present study, we show that a subset of agonistic EphA2 antibodies selectively bind epitopes on malignant cells, which are not available on nontransformed epithelial cells. We also show that such epitopes arise from differential cell-cell adhesions and that the stable intercellular junctions of nontransformed epithelial cells occlude the binding site for ligand, as well as this subset of EphA2 antibodies. Finally, we demonstrate that antibody targeting of EphA2 decreases tumor cell growth as measured using xenograft tumor models and found that the mechanism of antibody action relates to EphA2 protein degradation in vivo. Taken together, these results suggest new opportunities for therapeutic targeting of the large number of different cancers that express EphA2 in a manner that could minimize potential toxicities to normal cells.

INTRODUCTION

The EphA2 receptor tyrosine kinase critically controls many aspects of cell behavior (1). In normal cells, EphA2 expression appears to be restricted to a intercellular junctions between epithelial cells, where it binds ligands that are anchored to the membrane of adjacent cells (2, 3). Thus, stable cell-cell adhesions are necessary for EphA2-ligand binding (4). Much knowledge of EphA2 function has been inferred from a series of recent studies using tumor cells. These studies suggest that ligand binding causes EphA2 to become enriched within cell-cell contacts between nontransformed epithelial cells (4). When localized to these sites, EphA2 becomes autophosphorylated, which initiates a cascade of downstream signals that ultimately serves to negatively regulate cell attachment to extracellular matrix adhesions and thereby inhibit cell growth and migration (4–6). As such, EphA2 may contribute to the well-known phenomenon of contact inhibition. Ligand binding also causes EphA2 to become internalized and degraded, which results in the maintenance of low-level EphA2 expression in normal epithelial cells (7, 8).

EphA2 is frequently overexpressed and functionally altered in many invasive cancers. For example, high levels of EphA2 have been documented in metastatic melanoma, as well as cancers of the mammary gland, prostate, colon, lung, kidney, and esophagus (6, 9–16). The highest levels of EphA2 are predominantly found on the most aggressive cells, which is consistent with evidence linking EphA2 with clinical features of metastasis (6, 9, 11). Additional supportive evidence demonstrated that overexpression of EphA2 is sufficient to confer tumorigenic and metastatic potential upon nontransformed epithelial cells (6).

The EphA2 on malignant cells also functions very differently from its counterpart on normal cells (17). In particular, the EphA2 in malignant cells retains enzymatic activity but is itself not tyrosine phosphorylated (4). This is important because high levels of unphosphorylated EphA2 promote tumor growth, survival, and invasiveness, causing EphA2 to function as a powerful oncoprotein in malignant cells (6, 8). These outcomes result, in part, from weakened intercellular adhesions between malignant cells, which destabilize EphA2-ligand binding (4). Consequently, EphA2 loses its capacity to negatively regulate cell growth and instead EphA2 redistributes across the cell surface and is linked with mechanisms that positively regulate tumor cell growth, survival, and invasion (1). Compounding this, reduced ligand binding decreases EphA2 turnover, thus allowing EphA2 to accumulate on the surface of malignant cells, where it additionally promotes malignant behavior (7, 17). Taken together, these frequent defects in EphA2 expression and function serve to increase metastatic character and favor malignant progression.

Recently, we demonstrated that changes in EphA2 expression and function provide a much-needed opportunity for therapeutic targeting of cancer cells (1). Specifically, monoclonal antibodies can mimic the biochemical actions of ligand and thereby redirect the EphA2 on tumor cells such that it negatively regulates tumor cell growth, survival, and invasion in vitro (8). In our present study, we demonstrate that unique aspects of cancer cell biology expose EphA2 epitopes on malignant cells that are not accessible on normal cells. We also confirm the therapeutic efficacy of antibody targeting of tumor cells and extend these findings to show that EphA2 antibodies are efficacious in vivo.

MATERIALS AND METHODS

Cells and Reagents. MCF-10A, A549, and MDA-MB-231 cells were cultured as described previously (8). Antibodies specific for the cytoplasmic domain of EphA2 (clone D7) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies specific for β-catenin were purchased from Transduction Laboratories (Lexington, KY).

Antibody Production. BALB/c ByJ and SJL mice (The Jackson Laboratory) were used for immunizations. One group of mice were immunized with 10 μg of EphA2 mixed with an equal volume of TiterMax Adjuvant. One hind leg of each mouse was immunized in the dorsal region on days 0, 7, 12, and 17. Popliteal and inguinal nodes were removed for fusion on day 15. A second group of mice were immunized on days 0, 21, and 42 with EphA2 in TiterMax. l.p. Serum titers were determined on day 48. Prefusion boost was performed 3 days before splenectomy. Fusions of splenocytes or lymph nodes were performed using NS-0 cells using ClonaseCell-HY (StemCell Technologies).

Immunofluorescence Microscopy. Monolayers of MCF-10A or MDA-MB-231 cells were cultured atop glass coverslips for at least 24 h at 37°C before staining. Cell monolayers were fixed in 4% paraformaldehyde (2 min, 25°C) before incubation with primary antibody (clone EA2, B233, or EphrinA1-Fc) for 30 min followed by subsequent staining with rhodamine-conju-

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gated goat antioimmune IgG or FITC-conjugated goat anti-human IgG (The Jackson Laboratory). For EGTA treatment, MCF-10A cells were incubated in 4 mM EGTA for 10 min on ice. After EGTA treatment, cells were stained with EA2 or EphrinA1-Fc on ice and returned to regular media at 37°C for 15 min to restore cell-cell adhesion. Then, cells were fixed and stained with labeled secondary reagents for analyses by immunofluorescence microscopy or suspended in Versene for analysis using flow cytometry.

Western Blot Analyses. Sample extraction, immunoprecipitation and Western blot analyses were performed as detailed previously (8). For all experiments, protein levels were measured using standard Coomassie assays (Pierce, Rockford, IL), and equal amounts of protein were resolved by SDS-PAGE. Sample extraction, immunoprecipitation and Western blot analyses were performed as detailed previously (8). For all experiments, protein levels were measured using standard Coomassie assays (Pierce, Rockford, IL), and equal amounts of protein were resolved by SDS-PAGE (10%) and transferred to Nitrocellulose (Schleicher and Schuell, Keene, NH). For studies using tumor-derived material, the samples were homogenized and sonicated before detergent solubilization. To confirm equal sample loading, the membranes were stripped and reprobed using β-catenin antibodies.

ELISA. EphA2-Fc (1 μg/ml) was immobilized onto ELISA plates overnight at 4°C. Then the antigen was incubated with 100 μl of 1 μg/ml primary antibodies (clone D7, EA2, and B233), followed by alkaline phosphatase-conjugated AffiniPure goat antioimmune IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and pNPP substrate (Pierce) and read at 405 nm. D7, an isotype control that recognizes an intracellular epitope on EphA2, was provided a negative control. For epitope mapping assays, 1 μg/ml EphrinA1-Fc was immobilized as described above and then incubated with 1.0 μg/ml EphA2-Fc, followed by subsequent incubation with 1 μg/ml competing antibodies (EphrinA1-Fc, EA2, and B233) and then stained as described above. EphrinA1-Fc provided a negative control for binding. For competition assay, 1 μg/ml immobilized EphA2-Fc was incubated with 0.1 μg/ml biotinylated-EA2 (EZ-Link Sulfo-NHS-LC-Biotinylation kit; Pierce) alone or with indicated concentrations of unlabeled EA2, B233, or EphrinA1-Fc before labeling with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc.). Unlabeled EA2 provided a positive control for competition.

Tumor Xenograft Models. A total of 5 × 10⁶ MDA-MB-231 or A549 cells were implanted s.c. or in the mammary fat pad of athymic (nu/nu) mice as described previously (6). Tumor measurements were obtained twice weekly using Vernier calipers, and tumor volume was calculated as follows: \( V = L \times W^2/2 \). After the tumors reached an average volume of 100 mm³, the studies with A549 and MDA-MB-231 were performed and analyzed identically. Animals were humanely euthanized when tumor volume exceeded 1000 mm³, a preestablished end point. Upon sacrifice, tumor samples were obtained, and detergent extracts were prepared for Western blot analyses as detailed above.

RESULTS

Certain EphA2 Epitopes Are Not Accessible on Nontransformed Cells. Monolayers of nontransformed (MCF-10A) or highly aggressive (MDA-MB-231) human mammary epithelial cells were fixed and incubated with different EphA2 antibodies, followed by labeling with fluorophore-conjugated secondary antibodies. Most EphA2 antibodies, represented by clone B233, reacted with both nontransformed and malignant cells (Fig. 1). EphA2 was enriched within sites of cell-cell contact on nontransformed cells and was diffusely distributed over the surface of malignant cells. In contrast, a subset of EphA2 antibodies, represented by clone EA2, stained the surface of breast (MDA-MB-231) and lung (A549) cancer cells but reacted weakly with nontransformed epithelial cells (e.g., MCF-10A). EA2 immunoreactivity was conspicuously absent from sites of intercellular contact. Comparable results were obtained using additional nontransformed epithelial cell models, including MCF-12A and MCF-10-2 (data not shown), indicating that the observed differences in immunoreactivity were not unique to MCF-10A cells. Moreover, we also found that EphrinA1-Fc, an engineered form of ligand, similarly was excluded from binding nontransformed epithelial cells (Fig. 2).

We considered that genetic mutation of EphA2 could contribute to the decreased immunoreactivity of certain EphA2 antibodies such as EA2. However, DNA sequencing did not reveal mutations of EphA2 that could account for differential antibody reactivity (data not shown). Consistent with this, EA2 could recognize the EphA2 on nontransformed cells when used for immunoprecipitation of detergent lysates, which suggests that these epitopes simply are not accessible in intact monolayers. Furthermore, oncogene-transformed variants of MCF-10A (MCF-10ANeoST, MCF-10ANeoT, MCF-10A EphA2) regained reactivity with EA2 antibodies (Fig. 2 and data not shown). Taken together, these findings suggest that rather than representing a mutation of EphA2, certain epitopes (e.g., EA2) are selectively excluded by the normal architecture that typifies nontransformed epithelial cells but becomes accessible after malignant transformation.

The decreased immunoreactivity against nontransformed epithelial cells led us to ask if EA2 or B233 differ with regards to other binding characteristics. BiaCore and ELISA-based analyses both confirmed that EA2 and B233 specifically bind purified EphA2 extracellular...
Although the affinity of B233 (0.3 nM) for EphA2 was somewhat greater than that of EA2 (5 nM), these differences were insufficient to account for differential surface staining of nontransformed (but not malignant) cells, particularly at the relatively high antibody concentrations (>10 μg/ml) used for the cell staining studies. We could also exclude that EA2 binds an epitope that directly competes with ligand (EphrinA1). For example, EphrinA1 was immobilized onto ELISA plates to capture a purified form of the EphA2 extracellular domain. Captured EphA2 was readily detected using EA2 or B233 (Fig. 3B), which suggests that these antibodies bind epitopes that are distinct from the site on EphA2 where ligand binds. In a complementary experiment, EA2 was directly labeled as a probe for competition studies with B233 and EphrinA1-Fc (EA1-Fc), and the absence of B233 or EphrinA1 competition suggest that EA2, B233, and ligand bind different sites on EphA2 (Fig. 3C). Taken together, these results indicate that EA2, B233, and EphrinA1 recognize different epitopes on EphA2 and that the decreased EA2 binding to nontransformed cells does not represent competition with endogenous ligands.

**Differential Epitope Display Requires Stable Cell-Cell Contacts.** Previous studies have demonstrated that in nontransformed epithelial cells, stable cell-cell contacts favor ligand binding, whereas in malignant cells, unstable cell-cell contacts decrease ligand binding (4). Because the studies above excluded that EA2 binds an epitope that is occluded by ligand, we postulated that stable cell-cell contacts might have conferred steric hindrances that occlude EA2 binding. In particular, elements of the cell-cell contact itself might have imposed steric constraints that are absent in malignant cells. As an initial test of this hypothesis, the intercellular contacts by monolayers of MCF-10A cells were disrupted by calcium chelation, which destabilizes E-cadherin-mediated adhesions (18). Indeed, the addition of EGTA to cell culture medium was sufficient to permit EA2 staining of nontransformed cells (Fig. 4). As a control, we found that EGTA also allowed exogenous ligands (EphrinA1-Fc) to bind the EphA2 on nontransformed cells. As a more objective measure of antibody binding, a parallel study was performed using flow cytometry analysis. This analysis provided an objective confirmation of fluorescence microscopic studies and verified that EGTA treatment increased EA2 binding to the cell surface of MCF-10A but not MDA-MB-231 (Fig. 5). EGTA did not alter B233 binding to EphA2 as measured using either immunofluorescence microscopy or flow cytometry (data not shown).
Differential EphA2 epitope display on cells

Agonistic EphA2 Antibodies Are Efficacious in Vivo. We then evaluated the biological consequences of EphA2 antibodies in vitro and in vivo. Neither EA2 nor B233 altered the phosphorylation, degradation, or subcellular localization of EphA2 on nontransformed cells as measured by Western blot analyses and immunofluorescence microscopy (data not shown). Nor did these antibodies impact the growth or survival of nontransformed epithelial cells. In contrast, in vitro assays demonstrated that both EA2 and B233 caused the EphA2 on malignant cells to become phosphorylated and degraded (Fig. 6).

The potential efficacy of agonistic EphA2 antibodies has been inferred from biochemical and in vitro studies (8), although no evidence of in vivo activity has yet been reported. Thus, we asked if such antibodies would be efficacious in vivo. For our initial studies, MDA-MB-231 breast cancer cells were implanted in the mammary fatpad of athymic (nu/nu) mice. These cells were allowed to establish tumors in vivo ($V_0 = 100 \text{ mm}^3$) before randomization and treatment with EphA2 antibodies or a matched isotype (IgG1, 1A7; a negative control). The antibodies (6 mg/kg) were administered i.p. twice a week for 3 weeks. EA2 decreased tumor growth rates in vivo relative to the matched isotype controls (Fig. 7A).

A variety of controls were then performed to verify the observation that EphA2 antibodies could decrease tumor growth in vivo. For example, EA2 antibodies were also found to be efficacious for MDA-MB-231 cells that had been implanted s.c. (data not shown). The efficacy of EA2 for s.c. tumors was reduced as compared with studies using orthotopic models, which is consistent with similar findings with most cancer therapies. We also confirmed that EA2 could inhibit the growth of other EphA2-expressing tumor cells, including A549 lung cancer cells ($V_0 = 200 \text{ mm}^3$; Figs. 7B and 8B) and EphA2-transfected MCF-7 breast cancer cells (data not shown). As an additional control, we confirmed that we could also achieve tumor inhibition using other agonistic EphA2 antibodies such as B233 (data not shown), thus indicating that the tumor-inhibitory effects were not restricted to EA2.

On the basis of evidence that antibody efficacy in vitro relates to EphA2 turnover (8), we then asked whether EphA2 antibodies alter EphA2 protein levels in vivo. The tumors from the studies above were excised, and a portion of the tumors were homogenized in nonionic detergent. Equal amounts of tumor lysate (50 μg) were then resolved by SDS-PAGE and subjected to Western blot analyses with EphA2 antibodies. Although tumors from subjects treated with the
isotype control had high levels of EphA2, the tumor cells from EA2-treated subjects had lower levels of EphA2 (Fig. 8). These differences did not reflect differences in sample loading because equal amounts of protein were resolved and evaluated. It is also notable that animal E6, who had the highest amount of detectable EphA2 from the EA2-treated group, also had the largest residual tumor mass (Fig. 8 and data not shown). Identical results relating antibody efficacy to low levels of EphA2 were also achieved using B233, which also decreased EphA2 levels in vivo. Thus, these findings are consistent with evidence that agnostic antibodies function by inducing EphA2 turnover and extend these results by relating antibody efficacy in vivo with EphA2 degradation.

DISCUSSION

The major finding of our present study is that certain epitopes on EphA2 are selectively available on malignant cells but are not accessible on normal cells. We also show that the basis for these cryptic epitopes is that stable cell-cell contacts, which typify normal epithelial cells, prevent antibody accessibility. Finally, we demonstrate that agnostic EphA2 antibodies can block tumor growth in vivo and that antibody efficacy relates to decreased EphA2 protein levels.

The molecular basis by which certain epitopes are selectively accessible on tumors appears to relate to steric constraints imposed by stable cell-cell contacts. Our present findings suggest that cell-cell contacts could present a barrier that prevents EA2 from encountering its epitope. Because EA2 can accumulate into newly organized cell-cell contacts, we interpret this as evidence that the binding site for EA2 is not directly competitive with cell-cell contacts. Rather, cell-cell contacts likely impose a steric or conformational impediment to EA2 binding.

In this context, it is notable that cell-cell adhesions are often weakened or eroded in malignant cells (19, 20). Causes for these changes include genetic, epigenetic, and posttranslational modifications of E-cadherin, catenins, and other cell surface and cytoplasmic proteins that contribute to the stability of epithelial cell-cell adhesions (20). We cannot exclude that the EphA2 on normal cells might adopt certain unique conformations on malignant cells. Indeed, EphA2 demonstrates prominent differences in phosphotyrosine content and subcellular distribution when comparing nontransformed and malignant epithelial cells (4, 6, 11), and such differences could alter the availability of EA2 epitopes in malignant cells.

The observations reported herein have potential application to other antibody targets and for additional means of therapeutic targeting (small molecules, biologics). For example, epitope targeting could selectively target antigens on diseased cells based on altered conformation or accessibility and thereby limit potential toxicities to normal cells. Thus, epitope targeting could increase the efficacy of antibodies and may also facilitate higher tolerable doses of toxin-conjugated therapeutics.

Another novel aspect of our present study is the first demonstration that EphA2 antibodies can decrease tumor growth in vivo. The doses used herein (6 mg/kg) resulted in substantial decreases in tumor growth and are likely to be achievable in a clinical setting. If our present results can be translated into clinical efficacy, this would support the concept that EphA2 offers an attractive target for therapeutic targeting of the many different cancers that overexpress EphA2. In light of recent evidence linking EphA2 with many of the most aggressive forms of cancer, EphA2 antibodies could provide a much-needed therapeutic option for many intractable forms of the disease that are otherwise resistant to conventional therapies.

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