

EphB2 as a Therapeutic Antibody Drug Target for the Treatment of Colorectal Cancer

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Abstract

Analysis of human colorectal cancer specimens revealed overexpression of the EphB2 receptor tyrosine kinase. Monoclonal antibodies (MAbs) to extracellular sequence of EphB2 were raised and tested for activity against colorectal cancer cells. One of the MAbs, 2H9, effectively blocked the interaction of ephB2 with ephrin ligands and inhibited the resulting autophosphorylation of the receptor. However, this antibody did not affect the proliferation of cancer cells expressing ephB2. Immunocytochemical analysis revealed rapid internalization of the MAb 2H9 on binding ephB2, suggesting that target-dependent cell killing could be achieved with an antibody-drug conjugate. When MAb 2H9 was conjugated to monomethylauristatin E through a cathepsin B-cleavable linker, it specifically killed ephB2-expressing cancer cells *in vitro* and *in vivo*. Our results suggest that ephB2 is an attractive target for immunoconjugate cancer therapy.

Introduction

The eph receptor family constitutes the largest family of tyrosine kinase receptors in the human genome (reviewed in Refs. 1–3). The 14 human eph receptor tyrosine kinases are categorized by sequence identity into an A class and a B class with corresponding A-type and B-type ligands referred to as ephrins. Signaling can occur in a forward manner, in which the receptor tyrosine kinase is activated by the ligand, and in a reverse manner, in which the transmembrane ephrinB ligands are activated by interaction with receptors. Eph receptor ligand interactions have been implicated in a wide range of biological functions including axon guidance, tissue border formation, vasculogenesis, and cell motility (4–13). Many of these functions are consistent with evidence linking ephrin and eph receptor signaling to the alterations in the cytoskeleton (14, 15).

The precise signaling mechanisms that lead to the plethora of biological readouts attributed to eph receptor ligand interactions are only beginning to be understood. For example, association of ephA with the rho family exchange factor ephexin is consistent with the effects of these receptors on remodeling of the actin cytoskeleton observed in axonal growth cone collapse (16). The cytoplasmic regions of the activated eph receptors have been reported to interact with myriad familiar signaling molecules such as rasGAP, Src, Grb2, Abl, and phosphatidylinositol 3'-kinase (17–21). Although these molecules typically associate with growth factor receptors, the eph receptor tyrosine kinases are not known for promoting mitogenicity. Some weak proliferative responses have been observed, but the ephs have also been reported to inhibit mitogenic responses. The ephB2 receptor tyrosine kinase down-regulates the ras/mitogen-activated protein

(MAP) kinase signaling pathway and also inhibits the abl tyrosine kinase in endothelial and neuronal cells (20, 22, 23).

Despite the lack of evidence for eph receptor tyrosine kinases in mitogenesis, there have been numerous reports correlating their expression with cancer progression (1, 24–31). In particular, EphB2 expression has been observed in both gastric and colon cancers, neuroblastomas, small cell lung carcinoma, and melanoma and in a variety of human cancer cell lines (25, 32–34). Although there is a positive correlation between expression of eph receptor and cancer, it is not clear how the activity of the eph receptor could contribute to tumor progression. The potential influence of eph receptors on cell adhesion, motility, guidance, and position has been considered as a mechanism by which they could exacerbate tumorigenesis (1). Recently, ephB2 and ephB3 were implicated in controlling the segregation and position of cells along the crypt-villus axis in the intestine (35). This same study identified ephB2 as a target of the wnt signaling pathway, which is hyperactivated by genetic defects associated with the majority of colorectal cancers. Thus, overexpression of ephB2 in colorectal cancers is consistent with its activation by wnt signaling.

We have examined the expression of ephB2 in normal and diseased tissues obtained from numerous organs throughout the human body. The highest level of expression was observed in colorectal cancers, implicating ephB2 as a potential drug target in this disease. However, a monoclonal antibody (MAb) that neutralizes the activation of ephB2 by ephrin ligands did not affect the tumorigenic potential of colon cancer cells expressing ephB2 *in vitro* or *in vivo*. By contrast, an ephB2 antibody-drug conjugate capable of killing cells on internalization was highly effective in killing colon cancer cells in culture and the tumors derived from them *in vivo*.

Materials and Methods

Antibodies and Recombinant Proteins. Monoclonal anti-phosphotyrosine conjugated with horseradish peroxidase was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-epidermal growth factor (EGF), EGF, phospho-p44/42 MAP kinase, and p44/42 MAP kinase were purchased from Cell Signaling Technology (Beverly, MA). Antibody to the GD epitope and the fusion proteins ephrin-B1-Fc and ephrin-B2-Fc were produced at Genentech (South San Francisco, CA).

Cell Lines and Plasmids. The human colon adenocarcinoma cell lines SW480, SW620, and Colo 205 and the fibrosarcoma cell line HT1080 were obtained from the American Type Culture Collection (Manassas, VA). The HT1080-epHB2 and HT1080-GD cell lines were generated by cotransfection with a SV40-driven vector encoding an NH₂-terminal GD epitope-tagged form of ephB2 or empty vector, respectively, and with a cytomegalovirus promoter-driven puromycin vector. Cells were selected in 1 μ g/ml puromycin. The SVT2-epHB2 cell line was established in the same fashion, except that mouse 3T3 cells were cotransfected with a cytomegalovirus promoter driving Neo, and cells were selected in Geneticin (Life Technologies, Inc.) at 400 μ g/ml. Cells were grown in high-glucose DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin-streptomycin (100 units/ml).

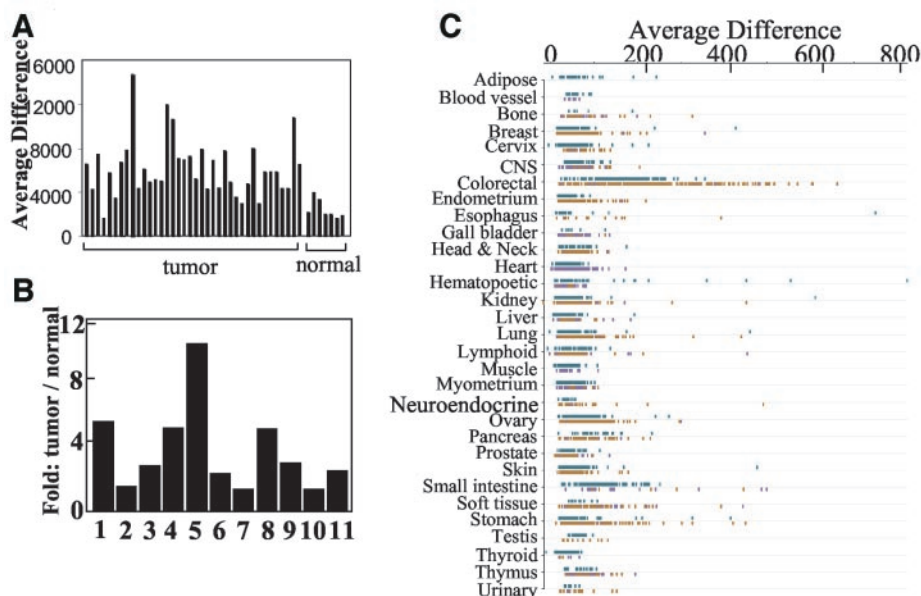
RNA Expression Analysis. For the analysis of tumor and normal colon tissue specimens (Fig. 1A), approximately 10 μ g of total RNA from each

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Fig. 1. Expression of ephB2 in cancer and normal human tissues. **A**, oligonucleotide microarray analysis was performed on RNA extracted from 38 human colorectal tumors and 7 specimens of normal human colonic mucosa. The average difference represents signal intensity (scaled to 1500). **B**, real-time PCR was performed on RNA extracted from 11 human colorectal cancer biopsy specimens and the corresponding patient-matched normal colon tissue specimens. **C**, oligonucleotide microarray analysis was performed on RNA extracted from 4841 human tissue samples. Each box in the plot provides signal intensity (average difference scaled to 100) for ephB2 for a sample of the indicated tissue. *Green boxes* are normal tissue, *red boxes* are tumors, and *blue boxes* represent other diseased tissues.



human tumor or normal colon tissue sample served as starting material for the preparation of probes required for oligonucleotide array analysis on the Affymetrix GeneChip. Probes were prepared according to the manufacturer's recommendations. After hybridization, the arrays were washed and stained with streptavidin-phycoerythrin and then scanned with the Gene Array scanner (Agilent Technologies). Default parameters provided in the Affymetrix data analysis software package were applied in determining the signal intensities, referred to as average differences. Sample normalization was done using global scaling as stated in the Affymetrix Expression Analysis Technical Manual, and a target intensity of 1500 was used to determine average difference expression values. For the analysis of ephB2 mRNA expression in multiple human tumor and normal biopsy samples (Fig. 1C), the Affymetrix data were obtained from Gene Logic, Inc. (Gaithersburg, MD). In the analysis shown, there are a total of 4841 samples [1808 normal samples (*green*); 1545 cancer samples (*red*); and 1488 non-cancer diseased samples (*blue*). Gene Logic data were also normalized using global scaling, but in this instance, the target intensity was 100. The Affymetrix data for ephB2 in Fig. 1, A and C, was generated from the U95 probe set ID 41678_at. Real-time PCR (TaqMan; Perkin-Elmer, Applied Biosystems) for ephB2 mRNA was performed using gene-specific primers (5'-CGA-GCC-ACG-TTA-CAT-CA-3' and 5'-TCA-GTA-ACG-CCG-TTC-ACA-GC) and probe (CCC-ACA-CCC-AGT-ACA-CCT-TCG-AGA-TCC). For *in situ* hybridization, a 458-bp ³³P-labeled antisense riboprobe was generated from an EphB2 PCR product using a primer with oligonucleotide sequence TCTGTCCATCTGTCCCGTCTCT and a sense control riboprobe with the primer GCCCTCTGGTGCTCTATCC.

Monoclonal Anti-EphB2 Antibodies. BALB/c mice (Charles River Laboratories, Wilmington, DE) were immunized with baculovirus-derived His₈-tagged EphB2 receptor diluted in Ribi adjuvant (Corixia, Hamilton, MT) twice a week, via footpad, five doses. B cells from lymph nodes were harvested from five mice demonstrating high serum titers and were fused with mouse myeloma cells (X63.Ag8.653; American Type Culture Collection). After 10–14 days, the supernatants were screened for antibody production by direct ELISA and by flow cytometry on HT1080-GD and HT1080-EphBR cells. Positives were subcloned twice to achieve monoclonality. For large-scale production of purified antibody, hybridoma cells were injected i.p. into pristine-primed BALB/c mice. The ascites fluids were pooled and purified by protein A affinity chromatography (Pharmacia Fast Protein Liquid Chromatography; Pharmacia, Uppsala, Sweden).

Immunological Procedures. The analysis of ephB2 activation by soluble ephrin-Fc ligand was performed by stimulating the SVT2-ephB2 cell line with purified Fc-ephrinB2 (5 μg/ml, 15min) followed by cell lysis in radioimmuno-precipitation assay buffer [50 mM Tris, 150 mM NaCl, 1% deoxycholate, 1% NP40, 2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and complete proteinase inhibitor mixture (Roche Molecular Biochemicals)]. Ten

μg of anti-GD MAb were added to the lysates, followed by protein G-agarose (Life Technologies, Inc.), and incubated overnight at 4°C. The immunoprecipitates were recovered, washed with lysis buffer, and subjected to SDS-PAGE and immunoblotting. Blots were incubated with 1 μg/ml anti-phosphotyrosine mouse MAb conjugated with horseradish peroxidase (Santa Cruz Biotechnology) or anti-GD mouse MAb. Goat antimouse horseradish peroxidase was used as a secondary antibody, and the blots were washed and developed using the Enhanced Chemiluminescence system (Pierce). Analysis of MAP kinase activation was carried out using the HT1080-EphB2 cell line. Cells were serum starved for 12 h and either left untreated or stimulated with EGF 100 ng/ml in the absence or presence of 5 μg human Fc-ephrinB2/ml. Cell lysates were equalized for protein concentration, subjected to SDS-PAGE, and immunoblotted with anti-phospho-EGF receptor or phospho-MAP kinase antibody.

For flow cytometry, cells were grown to 90% confluence and removed from plates using Cell Dissociation Buffer (Invitrogen). Cells were washed and resuspended in fluorescence-activated cell-sorting buffer (PBS with 1% BSA) and incubated for 45 min with anti-EphB2 MAb 2H9 or anti-GD antibody (Genentech) followed by 30-min incubation with antimouse secondary antibody conjugated to phycoerythrin. Analysis was performed on FACScan.

Antibody Binding and Internalization. Purified MAb 2H9 was iodinated using the lactoperoxidase method, and the radiolabeled antibody was purified from free ¹²⁵I-Na by gel filtration chromatography using a Pharmacia PD-10 column. Assessment of internalization was carried out essentially as described previously (36). Cells were incubated with iodinated antibody on ice and then shifted to 37°C for 4 h, followed by an acid/salt/urea incubation at 4°C to dissociate surface-bound ligand. Total surface-bound and internalized antibody was determined by scintillation counting. Internalization of EphB2 was also assessed by immunofluorescence staining of cells.

Immunohistochemical staining of human colon tumor sections with anti-ephB2 antibody was performed on frozen tissue sections. Sections containing malignant epithelial cells of a colorectal adenocarcinoma were incubated with primary antibody 2H9 at a concentration of 5 μg/ml, followed a biotinylated horse antimouse IgG affinity-purified antiserum. As control, an adjacent section was incubated with an irrelevant primary antibody and counterstained with hematoxylin.

Slides containing HT1080-ephB2 cells were incubated with 1 μg/ml 2H9 antibody for 30 min on ice and then shifted to 37°C in a CO₂ incubator for 1 h. The slides were washed in PBS, fixed in 3% paraformaldehyde, and incubated with rhodamine-conjugated antimouse IgG antibody (Jackson ImmunoResearch Laboratories) at a 1:200 dilution for 20 min at room temperature. The number of cell surface MAb 2H9 binding sites was estimated by incubating cells for 4 h on ice with a fixed concentration of ¹²⁵I-labeled MAb 2H9 combined with

increasing concentrations of unlabeled MAb 2H9, essentially as described previously (37).

Preparation of Anti-EphB2-Valine-Citrulline (vc)-Monomethylauristatin E (MMAE) Immunoconjugate. The conjugation of the anti-EphB2 antibody 2H9 and control anti-interleukin (IL)-8 antibody with MMAE was performed by Seattle Genetics Inc., as described elsewhere (38).

In Vitro Tumor Cell Killing Assays. The HT1080-EphB2 cell line or the vector control cell line were added to each well of 96-well microtiter plates at 1.5×10^3 cells/well, 100 μ l/well, and incubated overnight at 37°C in a humidified atmosphere of 5% CO₂. Cells were exposed to various concentrations of MAb 2H9-vc-MMAE or MMAE-vc-anti-IL-8 based on 1:3 serial dilutions. After incubation for 48 h, Cell Titer-Glo reagent (Promega, Madison, WI) was added to the wells at 100 μ l/well, and after a 10-min incubation at room temperature, the luminescent signal was recorded.

In Vivo Tumor Growth Assays. Female nude mice (Charles River Laboratories, Hollister, CA) were maintained in accordance with the guide for the care and use of laboratory animals. HT1080-EphB2 and HT1080-GD cells were harvested, resuspended in PBS, and injected s.c. into the right and left flanks (1×10^6 cells/flank), respectively, of 6–8-week-old mice. When tumors reached approximately 100 mm³, animals were dosed i.p. with 0.2 ml of native EphB2 MAb or MMAE-vc-2H9 or MMAE-vc-anti-IL-8 once a week i.v. at a final dose of 3 mg/kg body weight. The tumor volumes were determined by measuring the length (*l*) and width (*w*) and calculating the volume ($V = lw^2/2$) as described previously. Assays with the CXF1103 tumor line were performed by Oncotest GmbH (Feiburg, Germany). Affymetrix oligonucleotide array analysis was performed on tumors from the Oncotest collection, which demonstrated expression of ephB2 in CXF1103. This was confirmed by real-time PCR and immunohistochemistry. CXF1103 is a human colon tumor estab-

lished by serial passage in nude mice. Groups of 10 nude mice of NMRI background received s.c. tumor implants to obtain 30 mice bearing tumors of similar sizes for randomization. Tumors were grown to an average size of 100–200 mg, whereupon treatment with vehicle control, control antibody conjugate anti-GD-vc-MMAE, or anti-EphB2R antibody conjugate 2H9-vc-MMAE was initiated by i.v. injection. Antibody conjugates were administered at 3 mg/kg body weight at 7-day intervals for 3 weeks.

Results

In an effort to identify cell surface antigens expressed on human colorectal tumors, we performed oligonucleotide-based microarray expression analysis on 38 human colorectal tumors and 7 normal colon biopsy samples. Data mining revealed that ephB2 was overexpressed by 2–6-fold in the majority of the tumors relative to the average expression level of the normal samples (Fig. 1A). To confirm the overexpression in colon cancers, we performed real-time PCR (TaqMan) on 11 additional human colorectal tumors, each referenced to a patient-matched normal colon sample. These data were highly consistent with the microarray data (Fig. 1B). On mining of a larger database, containing microarray data from over 4800 human biopsy samples, we noted that ephB2 mRNA was preferentially expressed in intestinal tissue, with increased expression in colorectal, soft tissue, and gastric cancers relative to numerous other tissues (Fig. 1C). We also examined ephB2 mRNA expression by *in situ* hybridization on tissue microarrays. By this method, the only normal human tissues in

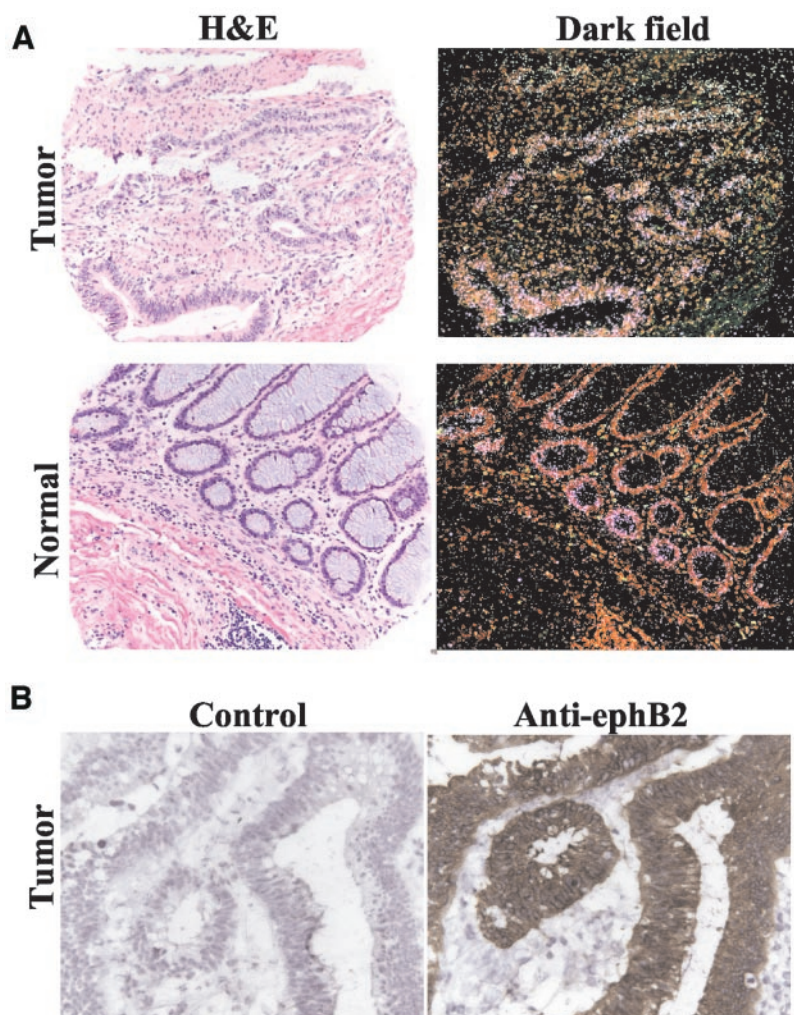


Fig. 2. EphB2 expression in human colon tissues. A, *in situ* hybridization. EphB2 transcript is detected as deposited silver grains in the dark-field images, and the corresponding bright-field images were generated by staining the sections with H&E. Top and bottom panels are from colon tumor and normal colon tissues, respectively. B, immunohistochemistry. Frozen serial tissue sections of a human adenocarcinoma were stained with ephB2-specific monoclonal antibody 2H9 (right panel) or with control antibody (left panel) and counterstained with hematoxylin.

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which ephB2 was detected were the colon and small intestine. *In situ* hybridization was also carried out on a gastrointestinal tissue microarray containing samples from a variety of cancers. Here ephB2 expression was seen in 12 of 18 primary colonic adenocarcinomas, 6 of 8 metastatic adenocarcinomas to the liver, 2 of 9 primary gastric carcinomas, and 1 of 4 esophageal carcinomas. No expression was observed in four of four pancreatic adenocarcinomas. Examples of expression in normal colonic mucosa and colorectal cancer by *in situ* hybridization are shown Fig. 2A. A MAb raised against human ephB2 also reacted strongly with tissue sections obtained from human colon adenocarcinomas (Fig. 2B).

The overexpression of ephB2 in colorectal cancers prompted us to raise MAbs against the extracellular sequence of the receptor. Several hybridomas were cloned that expressed MAbs reactive with the purified ephB2 immunogen and with the full-length ephB2 by flow cytometry (fluorescence-activated cell sorting) of cells expressing ephB2 (data not shown). MAbs from positive hybridomas were purified and compared for reactivity against colorectal cancer cell lines that endogenously express ephB2 and against cell lines engineered to overexpress the receptor (data not shown). A MAb designated 2H9 performed well in these assays, and the remainder of this study will focus on the application of this purified antibody.

The tyrosine kinase activity of ephB2 can be activated on ligation of the receptor with ephrinB ligands (12). This was observed when we incubated the murine 3T3 cell line expressing human ephB2 with a purified Fc-ephrinB fusion protein (Fig. 3A). We then tested the effects of MAb 2H9 in this assay. The tyrosine autophosphorylation of

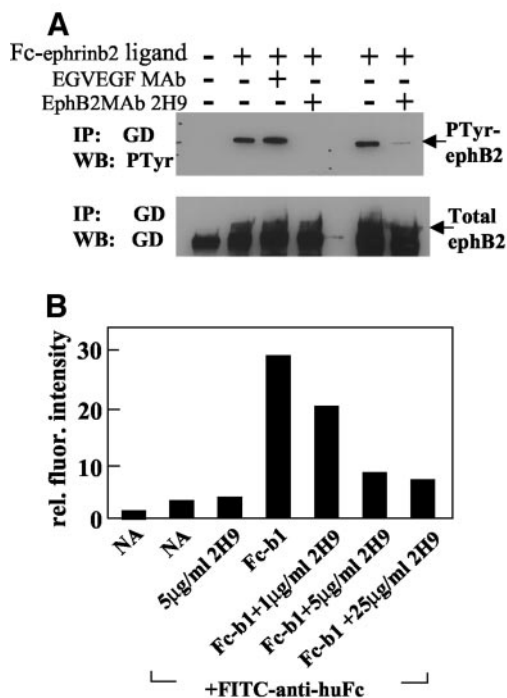


Fig. 3. Antagonism of ephrin-EphB2 interaction by monoclonal antibody (MAb) 2H9. A, the SVT2-ephB2 cell line was incubated with purified Fc-ephrinB2 fusion protein, and the EphB2 was recovered from cell lysates by immunoprecipitation (IP) through the GD epitope tag fused to its NH₂ terminus. Western blotting (WB) was performed with anti-phosphotyrosine (PTyr; top panel), to detect phosphotyrosine, and anti-GD (bottom panel), to detect total ephB2 protein. During ligand incubation, MAb 2H9 or control antibody (EGVEGF MAb) was included in the culture medium. B, binding of purified human Fc-ephrinB1 fusion protein to HT1080 cells overexpressing ephB2 was assessed by flow cytometry using human anti-Fc conjugated to FITC. Relative fluorescence intensity was then measured in the presence of increasing concentrations of MAb 2H9. As controls, Fc-ephrinB1 was omitted in the presence or absence of MAb 2H9 or secondary antibody.

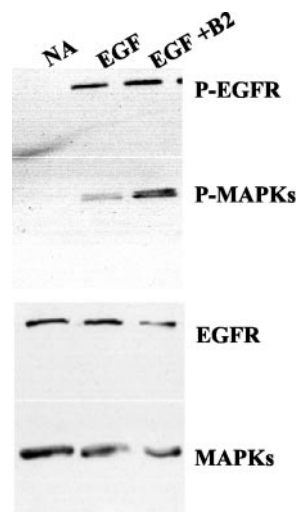


Fig. 4. Effect of Eph2 activation on the mitogen-activated protein kinase pathway. The HT1080 cell line overexpressing ephB2 was serum starved overnight and then treated with epidermal growth factor (EGF), EGF plus Fc-ephrinB2, or left untreated (NA). Lysates were subjected to immunoblotting with antibodies specific to phosphorylated forms of the EGF receptor and mitogen-activated protein kinase (top panel). A separate immunoblot was performed to determine total amounts of the two proteins (bottom panel).

ephB2 by Fc-ephrinB2 was inhibited when the cells were preincubated with MAb 2H9, whereas a control antibody had no effect (Fig. 3A). The mechanism by which MAb 2H9 inhibits ephB2 activation was investigated and found to involve competitive inhibition of ligand binding. This was determined by performing flow cytometry on cells incubated with the Fc-ephrinB1 ligand after their incubation with or without MAb 2H9. In this experiment, a positive fluorescence-activated cell-sorting signal results from the specific binding of FITC-conjugated antihuman Fc antibody to the bound Fc-ephrinB1 ligand. Increasing amounts of MAb 2H9 resulted in a corresponding decrease in Fc-ephrinB1 ligand binding (Fig. 3B). The loss of binding is not due to antibody-mediated receptor uptake because all incubations were performed on ice, where receptor internalization is minimal. Thus, MAb 2H9 inhibits the binding of ephrin ligands to the ephB2 receptor.

We next sought to determine whether MAb 2H9 was able to affect the mitogenic or tumorigenic potential of cancer cells. However, MAb 2H9 did not exhibit any specific effect on the growth of various cell lines *in vitro* that express the receptor (data not shown). An inhibition of cell growth might not be expected, however, because eph receptors are not considered to be mitogenic and have even been reported to interfere with mitogenic signaling as assessed by the activity of the MAP kinase pathway (22, 23). To assess this in a tumorigenic cell line strongly responsive to EGF, we generated stable clones of the fibrosarcoma cell line HT1080 overexpressing ephB2 (HT1080-ephB2). We found that ephrinB2 did not inhibit activation of the MAP kinase pathway by EGF in these cells but rather appeared to enhance it slightly (Fig. 4).

Regardless of the manner by which eph receptor activation might influence growth factor signaling, any antagonism by MAb 2H9 would still require the presence of ephrins because MAb 2H9 competes with their binding to ephB2. Although we determined that the HT1080 cell line produces ephrinB1 transcript, we do not know whether functional protein is released into culture medium at levels sufficient to activate the receptor. We therefore tested the 2H9 antibody in a more biologically relevant setting by growing the HT1080-ephB2 cell line as a tumor xenograft in nude mice that were then given MAb 2H9 at a dose of 10 mg/kg body weight twice per week.

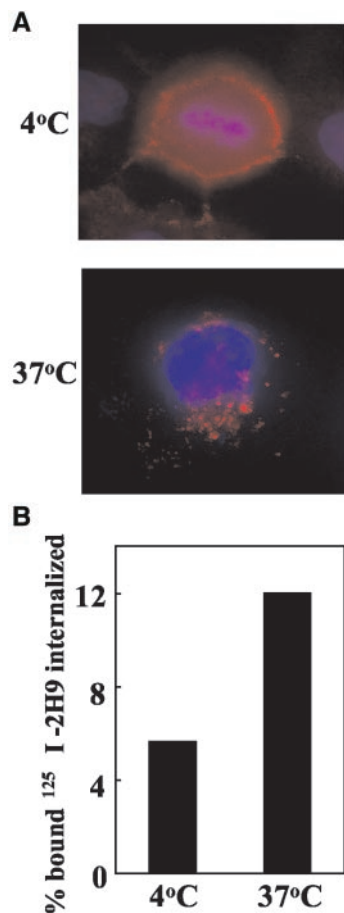


Fig. 5. Internalization of ephB2 monoclonal antibody (MAb) 2H9. A, MAb 2H9 was incubated on ice with HT1080 cells overexpressing ephB2. The cells either remained on ice or were shifted to 37°C for 1 h before fixing and staining with secondary antibody. B, MAb 2H9 was labeled with ¹²⁵I and incubated with HT1080-ephB2 cells on ice. Cells remained on ice (4°C) or were shifted to 37°C for 1 h, and then the percentage of internalized ¹²⁵I was determined by scintillation counting.

However, treatment with MAb 2H9 did not result in any significant effect on the rate of tumor growth in this assay (data not shown).

In some cases, naked antibodies directed against cell surface antigens have proven to be effective cancer therapies (39). Although it is still not clear why such antibodies are effective, whereas others are not, it likely relates to the specific function of the target antigen, and the effect the antibody has on it. In the event that naked antibodies fail, a viable alternative is to arm the antibody in a manner that delivers a drug to the targeted cells. In this approach, one is taking advantage of the antigen as a specific marker on the cancer cells, with less regard to the biological function of the antigen. These strategies sometimes rely on the uptake or internalization of the antigen-antibody complex such that drug is preferentially released inside of the cancer cells. To this end, we analyzed the internalization of MAb 2H9 after EphB2 binding. HT1080-ephB2 cells were incubated with MAb 2H9 on ice for 30 min and then shifted to 37°C for 1 h before fixation and staining with secondary antibody. Compared with cells kept on ice for the course of the experiment, the cells shifted to 37°C contained significant amounts of internalized antibody (Fig. 5A). We also examined MAb 2H9 uptake by incubating cells with ¹²⁵I-radiolabeled antibody at 4°C. The amount of ¹²⁵I-labeled MAb 2H9 that was internalized after cells were shifted to 37°C for 1 h was approximately double that of cells maintained at 4°C (Fig. 5B). Thus MAb 2H9 is readily internalized on binding to ephB2.

The uptake of MAb 2H9 suggested that it might effectively kill

cancer cells expressing ephB2 if conjugated to a potent drug. Therefore, the MAb 2H9 was covalently coupled to the drug MMAE through the peptide vc linker that is susceptible to cleavage by cathepsin B (39). On cleavage by cathepsin B, active MMAE is released, disrupting the dynamics of tubulin polymerization in the cell. We first tested the MMAE-vc-2H9 *in vitro* by treating the HT1080 cancer cells with increasing concentrations of antibody. Both the vector control HT1080 cell line (HT1080-GD) and the clonal derivative overexpressing ephB2 (HT1080-ephB2) were killed by MMAE-vc-2H9 at significantly lower concentrations relative to control antibody conjugate MMAE-vc-anti-IL-8 (Fig. 6). No effect was seen with underivatized MAb 2H9 up to a concentration of 10 mg/ml.

Although both the vector control and EphB2-expressing HT1080 cells were killed by MMAE-vc-2H9, there was an approximate 100-fold difference in apparent IC₅₀ values between these two cell lines. The relative number of MAb 2H9 binding sites/cell is therefore a likely determinant in the differential sensitivity of these two cell lines to MMAE-vc-2H9. This might reflect more efficient antigen cross-linking due to higher receptor density that could enhance internalization. To relate cell killing to receptor copy number, quantitative binding assays were performed using MAb 2H9 on the HT1080-GD and HT1080-ephB2 cell lines. By Scatchard analysis, approximately 71,000 and 308,000 copies/cell for these two lines, respectively, were estimated (Fig. 7A). For both cell lines, the apparent dissociation constant for MAb 2H9 was approximately 4 nM. The difference in ephB2 copy number was also evident from the relative fluorescence intensity observed when flow cytometry was performed with MAb 2H9 on the two cell lines (Fig. 7B). Also, the conjugation of MAb 2H9 with MMAE did not appreciably affect its binding properties to the HT1080 cells. The relative difference in receptor copy number on the two HT1080 cell lines was again evident from the binding of MMAE-

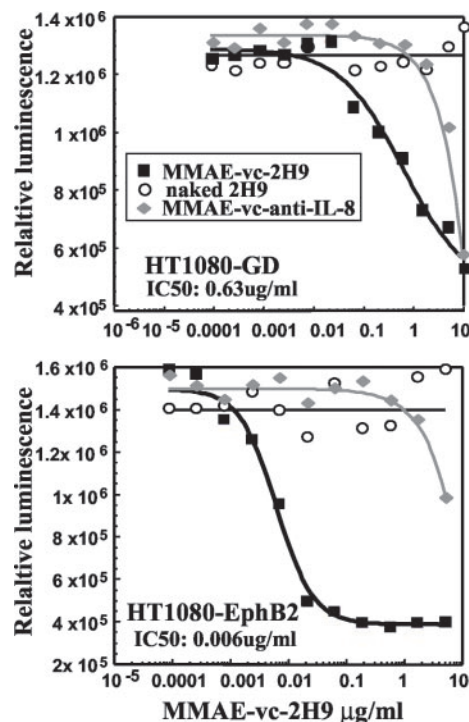


Fig. 6. *In vitro* tumor cell killing by monomethylauristatin E (MMAE)-valine-citrulline (vc)-2H9. The HT1080 cell line stably overexpressing ephB2 (HT1080-EphB2) or a vector control HT1080 cell line (HT1080-GD) was treated with increasing concentrations of naked monoclonal antibody 2H9, MMAE-vc-2H9, or MMAE-vc-anti-interleukin-8, and cell viability was measured after 2 days. The antibody concentration required to attain half-maximal killing is indicated as IC₅₀.

vc-2H9 (Fig. 7C). These results indicate that target copy number is an important factor in determining the efficacy of a drug-conjugated antibody. In the case of ephB2, a moderate difference in copy number resulted in a much greater difference in the IC₅₀ values determined *in vitro*.

MMAE-vc-2H9 was tested for *in vivo* efficacy by administration to nude mice bearing human tumor xenografts. Tumors were established by inoculating mice on one flank with the HT1080-GD cells and on the opposite flank with HT1080-ephB2 cells. Tumors were grown to a size of 100–200 mm³ before i.v. administration of MMAE-vc-2H9 once per week at a dose of 3 mg/kg. Additional animals were treated with vehicle control or with control antibody MMAE-vc-anti-IL-8 at 3 mg/kg. In this experiment, the HT1080-ephB2 cell line grew more rapidly than the HT1080-GD vector control cell line under control conditions. We do not believe this is due to overexpression of ephB2 because previous testing of HT1080-ephB2 clones did not reveal a reproducible effect on tumor growth rate. Nevertheless, both types of tumors responded well to treatment with MMAE-vc-2H9 relative to vehicle control and control antibody conjugate MMAE-vc-anti-IL-8 (Fig. 8A). Control animal groups were terminated between day 7 and 14 due to the aggressive growth of their tumors. Animals treated with MMAE-vc-2H9 were maintained out to 4 weeks, when treatment was discontinued. As an additional test of *in vivo* efficacy, a human colon tumor established by serial passage in nude mice was implanted s.c., and growth was measured during a treatment course identical to that described for the HT1080 model above. Significant growth retardation was again observed with MMAE-vc-2H9, relative to vehicle control or control antibody MMAE-vc-anti-GD (Fig. 8B). Overall, these results demonstrate specificity and efficacy of MMAE-vc-2H9 in *in vivo* tumor growth models.

Discussion

A major goal in the development of new therapies for cancer is the generation of drugs with enhanced therapeutic index relative to the first-line chemotherapeutics currently in use. It is widely anticipated that access to voluminous amounts of detailed molecular and genetic

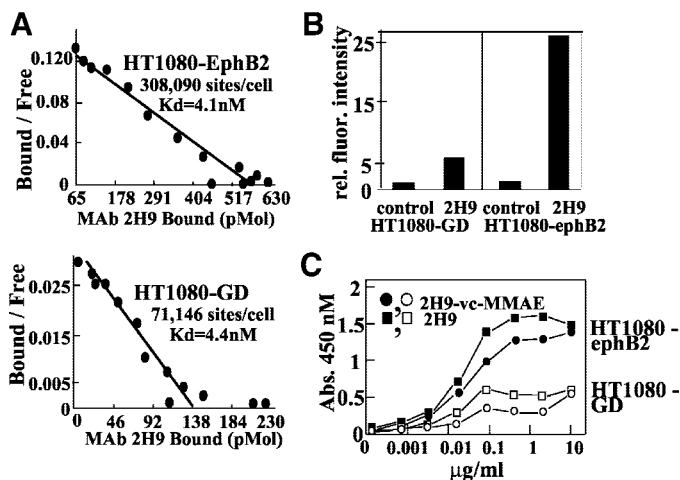


Fig. 7. Characterization of HT1080 cell lines and 2H9-valine-citrulline-monomethylauristatin E antibody. A, monoclonal antibody (MAb) 2H9 binding assays were performed on the HT1080-EphB2 and HT1080-GD cell lines. The estimated number of total binding sites and the dissociation constants (K_d) were determined by Scatchard analysis. B, flow cytometry was performed with MAb 2H9 on the HT1080-EphB2 and HT1080-GD cell lines. Relative fluorescence intensity is presented in the histogram for MAb 2H9 and for secondary antibody only (control). C, saturation binding was performed on the HT1080-EphB2 and HT1080-GD cell lines with both naked MAb 2H9 and monomethylauristatin E-valine-citrulline-2H9. Relative binding was determined using a horseradish peroxidase-conjugated secondary antibody, and absorbance was read at 450 nm.

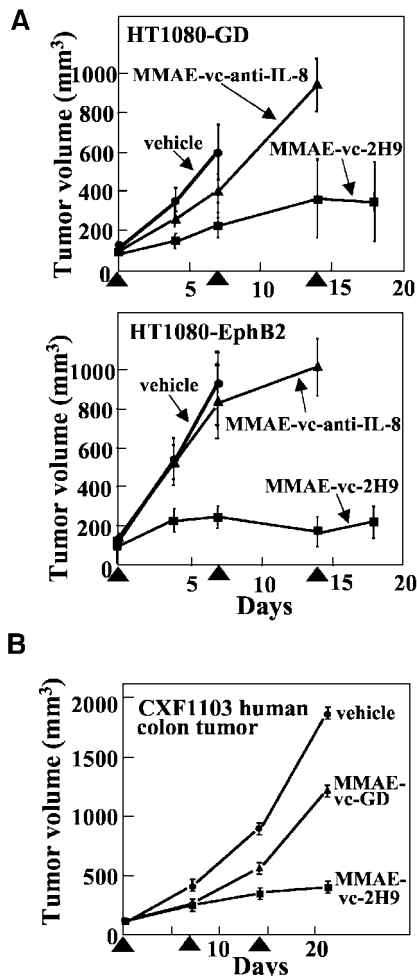


Fig. 8. Effect of monomethylauristatin E (MMAE)-valine-citrulline (vc)-2H9 on the growth of human tumor xenografts. A, nude mice were inoculated s.c. on one flank with HT1080-GD cells and on the other with HT1080-EphB2, and the resulting tumor xenografts were grown to an average size of 150 mm³ each. Ten animals in each group received vehicle control (●) or 3 mg/kg body weight of either MMAE-vc-2H9 (■) or MMAE-vc-anti-interleukin 8 (▲) once per week. B, the CXF1103 human colon tumor line was grown in nude mice that underwent a treatment protocol identical to that described for the HT1080 model. The control antibody was anti-GD conjugated to MMAE (MMAE-vc-anti-GD). Mean tumor volumes with SEs are presented.

information will lead to rational therapies designed to address specific cancers. Here we have exploited large-scale gene expression analysis to identify cell surface markers that are overexpressed in human colorectal cancer. Our analysis identified ephB2 as a receptor having a somewhat restricted tissue distribution with elevated expression in colorectal tumors. We confirmed this data by performing real-time polymerase chain reaction and *in situ* hybridization. Because MAbs have been proven successful in the treatment of cancer, we decided to test this approach with ephB2 as the drug target.

The MAbs generated against ephB2 were determined to bind with high affinity and specificity. However, although MAb 2H9 acted antagonistically with respect to EphB2 activation by ligand, it did not inhibit the growth potential of cells expressing this receptor. It is possible that ephB2R is not a requisite contributor to colorectal tumor progression. Despite ample correlative evidence linking the expression of Eph receptors and ligands to various human cancers, there is no direct evidence, such as mutations or gene amplifications, in support of any putative oncogenic function (1, 3). Conversely, ephB2 has been reported to inhibit mitogenic signaling in some settings (20, 22, 23). Although we did not observe this inhibition in cancer cell

lines, we also did not detect any growth advantage that correlated with receptor expression levels. Thus, overexpression or inhibition of ephB2 did not result in any measurable changes in tumor cell proliferation. Nevertheless, it is conceivable that EphB2 contributes to attributes of oncogenicity such as cell adhesion, invasion, and metastasis, which were not measured by our cell growth and tumorigenicity assays. Irrespective of target antigen function, it is conceivable that some naked antibodies could inhibit tumor growth by recruiting immune cells to antigen-bearing cancer cells (40). In particular, immune cells expressing Fc γ receptors have been reported to mediate antibody-dependent cytotoxic cell killing of human tumor xenografts in athymic mice (41). However, no such effect with MAb 2H9 was apparent in our tumor growth models.

In our second approach to MAb 2H9-mediated cell killing, we coupled the antibody to auristatin, a drug that interferes with the normal dynamics of tubulin polymerization (42). Drugs that operate by this mechanism of action have proven efficacy in cancer therapy and are routinely used as standard care agents (43). The safety margins of these drugs are not very broad, however, because their systemic delivery exposes normal proliferating cell populations to drug concentrations equivalent to that experienced by the tumor cells. Biasing the uptake of a drug into cancer cells relative to normal cells should thus improve on its therapeutic index. Accordingly, we have attempted to selectively deliver drug to those cells expressing the ephB2 target antigen. Indeed, cancer cells expressing ephB2 were killed by MMAE-vc-2H9 at concentrations far less than that required for killing by MMAE-vc-anti-IL-8, which did not bind to the cells. Similarly, MMAE-vc-2H9 was more effective at killing tumors expressing ephB2 than was the control antibody conjugate. Also, in both assays, the efficacy of MMAE-vc-2H9 was related to copy number of the antibody target, albeit to a lesser degree *in vivo*. Although the difference in copy number between the two cell lines tested was <5-fold, this resulted in a 100-fold difference in potency *in vitro*. However, this differential in potency was much smaller when tested against the corresponding tumor xenografts *in vivo*. It is unclear why the relative potency varied so greatly with respect to the two assays, but it might be related to differences in the kinetics of drug exposure under *in vivo* and *in vitro* conditions.

Based on the efficacy observed with MMAE-vc-2H9, ephB2 appears to be an attractive antigen for antibody-conjugate therapy. It should be pointed out that our testing was largely performed on a single tumorigenic cell line and on a single human colon tumor implant model. It is possible that other cancer cell lines or tumors might respond differently due to variations such as growth rate, drug resistance, and antibody internalization. Moreover, ephB2 is expressed on normal cells, and toxicity that might arise from targeting this antigen remains to be determined. The amino acid sequences of mouse and human ephB2 are 99% identical, and MAb 2H9 recognizes mouse ephB2. Therefore, it is likely that MAb 2H9 reacts with the ephB2 antigen that is expressed in mouse intestinal crypt cells (35). We have not observed any overt signs of drug-related toxicity in mice in the course of testing MMAE-vc-2H9 in the tumor models described in this study. However, additional studies in organisms more appropriate for testing drug toxicity will have to be performed.

Previous attempts at developing toxin-conjugated antibodies for cancer therapy have typically used potent proteinaceous bacterial toxins such as Ricin toxin A chain and *Pseudomonas* endotoxin (44, 45). Unfortunately, these toxins are immunogenic, promoting the rapid generation of antitoxin antibodies in the patient. More recently, organic compounds with favorable chemical and biological properties appropriate for conjugation and release from antibodies have been identified. The compounds can be coupled to antibodies through linkers designed to add additional specificity to the process of drug

delivery. In the case of MMAE-vc-2H9, the linker is cleaved by cathepsin B, which resides predominantly in the lysosome, thus providing some restriction on drug release to intracellular compartments (38). Therefore, the therapeutic index of such drugs is determined by tissue distribution of the target antigen, the mechanism of action of the drug itself, and the requirements set for release of active drug. We believe that the drug-linker combination used in this study represents a significant advance in this regard. With the gene expression tools and data available today, the ability to identify optimal target antigens has also been greatly improved.

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