Selective Targeting and Potent Control of Tumor Growth Using an EphA2/CD3-Bispecific Single-Chain Antibody Construct

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Abstract

The EphA2 receptor tyrosine kinase is frequently overexpressed and functionally altered in malignant cells and thus provides opportunities for selective targeting of tumor cells. We describe here the development of a novel, bispecific single-chain antibody (bscAb) referred to as bscEphA2xCD3. This molecule simultaneously targets EphA2 on tumor cells and the T-cell receptor/CD3 complex on T cells and possesses structural and functional characteristics of the recently developed BiTE technology. An EphA2-specific single-chain antibody was selected for recognition of an epitope that is preferentially exposed on malignant cells based on the concept of epitope exclusion; this was fused to a CD3-specific single-chain antibody to generate bscEphA2xCD3. The resultant bscAb redirected unstimulated human T cells to lyse EphA2-expressing tumor cells both in vitro and in vivo. In separate experiments, efficient tumor cell lysis was achieved in vitro at drug concentrations ≤1 μg/mL, at a low T-cell effector-to-tumor target cell ratio (1:1), and with tumor cells that possess few available binding sites (2,400 per cell) for bscEphA2xCD3. Time-lapsed microscopy revealed potent cytotoxic activity of bscEphA2xCD3-activated T cells against monolayers of malignant cells but not against monolayers of nontransformed EphA2-positive cells except at the edges of the monolayer where the target epitope was exposed. BscEphA2xCD3 was also efficacious in human xenograft mouse models modified to show human T-cell killing of tumors. Together, our results reveal opportunities for redirecting the potent activity of cytotoxic T cells towards tumor cells that express selectively accessible epitopes and establish EphA2-specific bscAb molecules as novel and potent therapeutics with selectivity for tumor cells. [Cancer Res 2007;67(8):3927–35]

Introduction

The EphA2 receptor tyrosine kinase is selectively overexpressed on many different cancers, and high levels of EphA2 have been observed on tumors of multiple lineages, including metastatic melanomas and on carcinomas of the breast, cervix, colon, esophagus, lung, ovary, prostate, and kidney (1). Retrospective analyses indicate that the highest levels of EphA2 expression are consistently found on the most aggressive cancers, and that EphA2 overexpression conveys an independent prognostic value for metastasis and patient survival (2–9). Consistent with this, ectopic overexpression of EphA2 is sufficient to confer tumorigenic and metastatic potential upon nontransformed epithelial cells (10).

EphA2 binds ligands, known as ephrins, which are anchored to the membrane of neighboring cells (11). Benign epithelial cells normally form stable linkages with adjacent cells, and the low levels of EphA2 that are present on benign epithelial cells are enriched within intercellular junctions (12). This localization favors stable ligand binding, and indeed, EphA2 on benign cells is autophosphorylated (12, 13). Malignant cells generally show unstable cell-cell contacts (14–16), and a consequence of this change is that the high levels of EphA2 on malignant cells fail to bind ligand and thus become diffusely distributed over the cell surface (12). Thus, EphA2 function is altered on malignant cells (17). Our earlier studies suggested that differential ligand binding might provide opportunities to identify specific residues that are uniquely exposed on malignant cells, and a subset of EphA2-specific monoclonal antibodies (mAb) were subsequently identified as binding selectively to epitopes on malignant cells (18).

mAbs have provided important new breakthroughs for cancer treatment. The high specificity of antibodies can enhance the targeting of diseased cells while minimizing the unwanted adverse effects on normal tissues. Receptor tyrosine kinases, including epidermal growth factor receptor and HER2, have been validated as particularly good targets for antibody-based therapy (19). Although mAbs have proven to be highly effective, there remain opportunities for further improvements (20). For example, relatively large amounts (mg/kg) of antibodies are generally necessary for therapy (21–23). Moreover, individuals who have certain polymorphisms in their Fc receptors seem to be less responsive to antibody-based therapies (24–26). In light of such potential limitations, we have sought to expand upon the principles validated by mAbs. To this end, we report herein the development and utilization of EphA2/CD3 bispecific single-chain antibodies (bscAb).

BscAbs of the BiTE class consist of two flexibly linked single-chain antibody fragments (scFv) that have the potential to redirect tumor resident and circulating T cells to lyse tumor cells (27). One scFv interacts with epitopes on tumor cells, whereas the other scFv binds an epitope on the T-cell receptor (TCR)/CD3 complex of T cells. This dual specificity seeds the formation of transient lytic synapses between T lymphocytes and malignant cells, which causes subsequent lysis of the malignant cells (28). Thus, BiTE molecules can redirect both CD4+ and CD8+ T cells to kill tumor cells in a serial fashion that is independent of the TCR-defined specificity and expression of human leukocyte antigens (HLA) on malignant cells (28–30). The concepts underlying the BiTE technology have
been successfully validated in preclinical studies with two different target antigens: CD19 (29–33) and EpCAM (34–37). A CD19/CD3-directed bscAb (referred to as MED1-538, MT103, or bscCD19xCD3) is currently being investigated in the clinic as a potential therapy for B-cell malignancies. Herein, we report the construction of an EphA2-specific bscAb that was designed to target epitopes that are uniquely exposed on malignant cells. We also show the specificity and potent efficacy to redirect T-cell lysis of the EphA2-specific bscAb in both cell culture and animal models and provide evidence that the epitope exclusion properties of the anti-EphA2 scFv are retained in the context of the EphA2-specific bscAb.

Materials and Methods

Selection, expression, and purification of bscEphA2xCD3. EphA2-specific bscAB molecules were constructed from the V\text{H} and V\text{L} domains that were cloned from the mouse hybridoma EdA2 (18) into the expression vector pEF-DHFR using the methods described previously (29). EdA2 binds to a unique human EphA2 epitope that is selectively available on malignant cells but not accessible on normal cells; the mAb blocks tumor growth in vivo (18).

EphA2-specific bscAB molecules of the BiTE class consist of a CD3\text{ε}-specific single-chain antibody derived from a "deimmunized" version of the mouse mAb L2K (30). The deimmunization process altered L2K amino acids using a procedure to maintain specificity of antibody binding and reduce the number of human T helper cell epitopes by elimination of MHC class II anchor amino acids within potential immunoreactive epitopes (38).

Chinese hamster ovary (CHO dhfr⁺ : American Type Culture Collection, McLean, VA) cells were transfected with the expression vectors encoding EdA2-based bscAb constructs that contained a COOH terminus hexahistidine tag, and stable cell lines were selected (29). CHO cell supernatants that contained secreted protein were centrifuged and enriched for bscAb molecules by immobilized metal affinity chromatography as described (36) and purified by gel filtration chromatography on a Sephacryl S200 HiPrep column (Amersham, Piscataway, NJ) equilibrated in PBS. Coomassie blue (Invitrogen, Carlsbad, CA)–stained SDS-PAGE and detection by Western blot confirmed the size, identity, and purity (>95%) of the isolated protein. Protein concentrations were estimated using a MicroBCA estimation by Western blot confirmed the size, identity, and purity (>95%) of the isolated protein.

Time-lapsed microscopy. Transformed A549 or MDA-MB-231 cells, or nontransformed MCF10A cells were seeded into a tissue culture–treated 48-well plate (BD Biosciences, San Jose, CA) for 24 h to establish an adherent cell monolayer. Directly before recording images, some monolayers were disrupted by scraping the wells with a pipette tip. Each well received effector cells in 400 μL of medium alone or mixed with bscAb molecules to a final concentration of 100 ng/mL. Stimulated human CD8+ T cells were used as the effector cells and added to an effector-to-target (E/T) ratio of 1:1 (intact monolayer panels) or 1:3 (disrupted Monolayer panels) with a negative control bscAb, positive control bscAb specific for a pancarcinoma antigen (EpCAM), or bscEphA2xCD3, in media containing 20 mmol/L HEPES to stabilize pH. Each plate was sealed with a transparent film (Fasson S695) to prevent evaporation and placed into a 37°C climate chamber on the stage of a Nikon microscope with an attached digital camera. Transmission light micrographs were recorded for a duration of 20 h (~1 picture per minute) and converted to an AVI video movie.

In vivo efficacy of bscEphA2xCD3 was evaluated in xenograft animal models that consisted of NOD/SCID mice that received a s.c. engraftment of 10⁶ cells and were randomized to treatment groups. Disease progression and tumor size were measured with a caliper or, in the case of large tumors, by computerized planimetry. All mice were observed daily for signs of distress, and if severe distress was observed, the mice were humanely euthanized.

Cell line and vector selection. CHO dhfr⁺ and the human cell lines SW-480 (colorectal adenocarcinoma), A375 (melanoma), PC3 (prostate adenocarcinoma), A549 (non–small cell lung alveolar adenocarcinoma), MDA-MB-231 (breast adenocarcinoma), MCF10A (breast epithelial), MDA-MB-468 (breast adenocarcinoma), and SK-MEL-28 (malignant melanoma) cell lines were obtained from the American Type Culture Collection and cultured in the recommended media. M14 (melanoma) was acquired from the National Cancer Institute Division of Cancer Treatment and Diagnosis cell line repository and cultured using the recommended media and conditions. The HeyA8 (ovarian carcinoma) cell line was a kind gift from Dr. Anil Sood, M.D. Anderson Cancer Center (39).

Immunohistochemical microscopy. Frozen human tissue sections were stained as described (40) with the human EphA2-reactive mAb, EdA2, to determine if the antibody specifically binds to normal tissue. Tissue sections were dehydrated, coverslipped, and imaged.

Surface plasmon resonance biosensor analysis. All studies were done using a Sensor Chip CMS (Biacore AB, Uppsala, Sweden) that contains a carboxymethyl dextran matrix and a Biacore3000 surface plasmon resonance biosensor (Biacore). Soluble CD3\text{ε} was covalently attached to the carboxymethyl dextran matrix using amine-coupling chemistry. A reference surface was created by omission of the CD3\text{ε} coupling step. EphA2-Fc was captured via a high-affinity interaction between the Fc portion of EphA2-Fc and a goat anti-human IgG (Fc) (KPL, Inc., Gaithersburg, MD). Goat anti-human IgG (Fc) was covalently attached to the carboxymethyl dextran matrix using amine-coupling chemistry. Two anti-human IgG (Fc)–specific surfaces were created. One of these surfaces was used as a reference surface, whereas the other surface was used to create an EphA2-Fc–specific surface. Different concentrations of bscEphA2xCD3 were prepared by serial dilution in PBS (pH 7.4), 0.15 mol/L NaCl, 3 mmol/L EDTA, 0.005% surfactant P20. BscEphA2xCD3 was injected in a serial-flow manner across the CD3\text{ε}-specific or EphA2-specific surface and its corresponding reference surface. Dissociation of bound bscEphA2xCD3 was monitored in the presence of PBS. Remaining bound material was removed with 10 mmol/L disodium tetraborate (pH 8.5), 1 mol/L NaCl (for CD3\text{ε}-specific surface), or 10 mmol/L glycine (pH 1.7; for EphA2-Fc–specific surface).

Cell surface antigen density. The number of EphA2 surface binding sites on HeyA8, SW-480, and A549 human carcinoma lines and M14 and A375 melanoma lines was estimated using Qifikit (DakoCytomation, Carpinteria, CA) as per manufacturer's instructions using the anti-human EphA2 antibody B233 (18). The number of surface bindings sites was estimated by nonlinear regression analysis from the bead calibration curve. Trypsin cleaves 10% to 20% of EphA2 from the surface of cells compared with using EDTA to remove adherent cells from tissue culture flasks (data not shown). Surface levels of EphA2 on trypsinized cells were equivalent if measured immediately after treatment with trypsin or after a 42-h incubation period at 37°C in non–tissue culture–treated 96-well plates.

Cytotoxicity assays. BscAb-mediated cytotoxicity was measured using either a standard chromium release assay or a flow cytometry–based assay. The chromium release assay and calculations were conducted as described (29). Flow cytometry–based redirected cellular cytotoxicity assays were carried out as described (30) except using CD3+ T cells enriched from human peripheral blood mononuclear cells (PBMC) as effector cells and various EphA2-positive human carcinoma cell lines, melanoma cell lines, or transfected CHO cells as a source of target cells. Target cells were labeled with 3,3'-dioctadecyloxacarbocyanine [DiOC6(3) or "DiO"; Invitrogen] green fluorescent membrane dye to distinguish them from PBMCs by flow cytometry after addition of propidium iodide. EC₅₀ values (estimated bscAb concentration at which half-maximal lysis of target cells occurred) were calculated using a four-variable nonlinear fit model.

Cell lines and culture. CHO dhfr⁺ and the human cell lines SW-480 (colorectal adenocarcinoma), A375 (melanoma), PC3 (prostate adenocarcinoma), A549 (non–small cell lung alveolar adenocarcinoma), MDA-MB-231 (breast adenocarcinoma), MCF10A (breast epithelial), MDA-MB-468 (breast adenocarcinoma), and SK-MEL-28 (malignant melanoma) cell lines were obtained from the American Type Culture Collection and cultured in the recommended media. M14 (melanoma) was acquired from the National Cancer Institute Division of Cancer Treatment and Diagnosis cell line repository and cultured using the recommended media and conditions. The HeyA8 (ovarian carcinoma) cell line was a kind gift from Dr. Anil Sood, M.D. Anderson Cancer Center (39).

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SW480 cells and unstimulated human CD3-positive T cells that has been previously described (34, 35). Tumor growth was measured at the indicated days with calipers in two perpendicular dimensions: tumor volume (cm$^3$) was calculated using the formula (width$^2$ × length) / 2 and reported as the arithmetic mean for each group and was used as a surrogate marker for treatment efficacy. Error bars in each graph represent the SE for the reported tumor volumes. The results are presented from one experiment and are representative of two separate studies.

At various time intervals after i.v. engraftment, mouse spleens were evaluated for the presence of viable human CD3$^+$ T cells. Briefly, anti-asialo GM1 antibody–treated SCID mice were administered 10⁷ human CD3$^+$ T cells by i.v. injection; the spleens of designated mice were collected, stained with APC-conjugated anti-human CD3 and propidium iodide, and analyzed by flow cytometry.

Results

**Generation of a bscAb with selectivity for EphA2$^+$ tumors.**

For bscAb construction, the anti-human EphA2 mAb EA2 was selected based on its ability to bind an epitope on EphA2 that is accessible on transformed cells and not on nontransformed cells (18). In light of the shown potency of other bscAb molecules of the BiTE class, we rationalized that epitope exclusion, in combination with EphA2 overexpression on tumor cells, should minimize potential binding and toxicity to benign tissues. To test for selective binding to tumors, the binding of EA2 to a panel of normal human tissues and cell lines was assessed by immunohistochemical analyses (Fig. 1). EA2 did not stain any of the 15 normal frozen human tissue sections, including tissues obtained from normal human heart, liver, lung, or spleen as shown in Fig. 1B. As controls, we verified that EA2 retained the ability to stain human EphA2-expressing PC3 prostate tumor cells, but not EphA2-negative breast adenocarcinoma–negative cells (Fig. 1A). An isotype-matched mAb (1A7) provided a negative control (data not shown). Surface plasmon resonance determined that the equilibrium dissociation constant ($K_D$) of the EA2 mAb for EphA2 was ~6 nmol/L.

A deimmunized derivative of the human CD3$\gamma$-specific murine mAb L2K called diL2K (30) provided the effector scFv for the EphA2-specific bscAb molecules. This antibody had deimmunized variable domains, which minimized a potential inactivation of resulting bscAb molecules by a neutralizing antibody response of the host. $K_D$ of mAb diL2K for CD3$\gamma$ was ~87 nmol/L as determined by surface plasmon resonance.

BscEphA2xCD3, the resultant bscAb constructed from EA2 and diL2K scFvs, redirected T cells to lyse MDA-MB-231 cells at a half-maximal concentration (EC$_{50}$) of 6 to 8 ng/mL. As additional evaluation criteria, stability in human sera, expression levels in cell culture supernatant, and binding affinities of the different bscAb molecules were analyzed. BscEphA2xCD3 was found to have stable biological activity in human plasma (100% active after 24 h as determined by an 18-h cytotoxicity assay and compared with untreated material), was produced at high yields, and showed an imbalance in affinities for EphA2 and CD3. BscEphA2xCD3 bound EphA2 with an apparent affinity of $K_D$ equal to 113 nmol/L, whereas CD3$\gamma$ was bound with an estimated $K_D$ of 400 nmol/L.

**Potent redirected lysis of EphA2-expressing cell lines by bscEphA2xCD3-activated T cells.** The biological activity of bscEphA2xCD3 was measured by an ex vivo cytotoxic assay in which SW480 human colon carcinoma cells provided targets for
human peripheral CD3+ T cells. In the presence of bscEphA2xCD3, previously unstimulated T cells were redirected to lyse SW480 tumor cells in a dose- and time-dependent manner (Fig. 2A–C), but as shown with SK-MEL-28 cells, bscEphA2xCD3 did not initiate redirected lysis of EphA2-deficient cells by T cells (Fig. 2A). We also examined the need for TCR/CD3 engagement by adding excess amounts of anti-CD3 IgG (DiL2K) to the cytotoxicity assay. The resulting decrease in potency confirmed the need of bscEphA2xCD3 to engage CD3 to stimulate T-cell lysis of target cells (Fig. 2B). Likewise, additional excess EphA2 antibody (EA2) decreased bscEphA2xCD3-mediated killing of target cells in a dose-dependent manner (Fig. 2C), and showed that T cell–mediated tumor cell lysis by bscEphA2xCD3 was dependent on the recognition of the EphA2 target. Finally, incubation of T cells and SW480 tumor cells in the presence of a bscAb that recognizes CD19, which is not expressed by SW480 tumor cells, did not promote killing of the SW480 cells despite having a CD3-binding arm similar to that of bscEphA2xCD3 (Fig. 2A).

To explore the kinetics and potency of bscEphA2xCD3, several variables of redirected tumor cell lysis were evaluated. An analysis of the time course of target cell killing revealed that in the presence of bscEphA2xCD3, there was limited lysis by unstimulated CD3+ T cells after 18 h, and that maximal killing (>80% lysis) occurred by 42 h (Fig. 3A). In most experiments, the magnitude of redirected T-cell lysis exceeded 80% of target cells. As an additional measure of potency, E/T cell ratio was investigated. E/T ratios of 1:1 to 20:1 still led to redirected lysis of SW480 tumor cells albeit at a reduced percentage (Fig. 3B). Notably, the estimated EC₅₀ values remained largely constant despite variations in incubation time (Fig. 3A; 1–9 ng/mL) or E/T ratio (Fig. 3B; 2–7 ng/mL).

Finally, tumor cell targets, which expressed different levels of EphA2 on the surface, were evaluated to determine whether there was a threshold of surface target density required for the activity of bscEphA2xCD3. Efficient redirected T-cell lysis was observed for all EphA2-expressing cell lines (Fig. 3C), including M14 cells, which expressed as few as 2,400 molecules of EphA2 per cell (Fig. 3D). The magnitude of lysis mediated by bscEphA2xCD3 was similar for target cells irrespective of their surface target density (Fig. 3D). However, the surface density of EphA2 on target cells did have an effect on the efficiency of redirected lysis. We observed a trend in which the potency of bscEphA2xCD3 increased as the number of EphA2 binding sites on the tumor cells increased (Fig. 3C). Together, these findings suggested that bscEphA2xCD3 can potently and specifically redirect unstimulated human T cells to lyse EphA2-expressing tumor cells, even when there are low levels of available binding sites on the tumor.

bscEphA2xCD3 selectively lysed malignant cells and spares nontransformed epithelial cells when the EphA2 epitope is occluded at cell-cell junctions. The EphA2-specific mAb EA2, from which bscEphA2xCD3 was derived, showed selective binding to tumor cells (i.e., epitope exclusion; ref. 18). Here, we investigated whether bscEphA2xCD3-mediated cytotoxic T-cell activity could differentiate between transformed (lacks contact inhibition; EphA2 expression over the entire surface of the cell) and nontransformed (maintains contact inhibition; EphA2 expression primarily within tight junctions) cells and thus determine if the epitope exclusion characteristic of EA2 mAb had been conserved in the generation of bscEphA2xCD3. A time-lapsed video microscopy study was undertaken to compare the reactivity of T cells in the presence of bscEphA2xCD3 against EphA2-positive, nontransformed epithelial

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**Figure 2.** Specificity of bscEphA2xCD3 in vitro cytotoxic activity. A, specificity of target cell lysis. CD3+ T cell–enriched PBMCs were incubated with EphA2-positive SW480 (● and ▲) or EphA2-negative SK-MEL-28 (○ and ▼) cells in the presence of serial dilutions of bscEphA2xCD3 (● and ▲) or bscCD19xCD3 (▲ and ▼) for 42 h at 37°C. B and C, parental antibodies to CD3 (DiL2K) and EphA2 (EA2) inhibit bscEphA2xCD3 lysis of EphA2-positive tumor cells. CD3+ T cells were incubated in 200 μL of culture medium with SW480 cells in the presence of serial dilutions of bscEphA2xCD3 alone (○), in the presence of 10 μg (▲) or 100 μg (●) DiL2K in (B) or EA2 in (C) for 42 h at 37°C. Percentage of lysed target cells. Points, mean of duplicate measurements; bars, SD. Note: the magnitude of lysis approaches 100% for each bscEphA2xCD3 curve.
cells (MCF10A) and against EphA2-positive malignant A549 or MDA-MB-231 cells. As expected from the cell lysis assays presented in Figs. 2 and 3, bscEphA2xCD3-activated T cells lysed A549 and MDA-MB-231 tumor cells all across the tumor cell monolayers that lead to clusters of highly activated T cells at sites where the monolayer was cleared (Fig. 4D). Cell lysis was further confirmed by positive immunofluorescence staining with propidium iodide only at the sites of T-cell attack (data not shown). Disruption of the monolayer did not alter the distribution or pattern of cytotoxic activity of T cells across the monolayer (Fig. 4A–D, disrupted monolayers). The specificity of tumor cell killing seen by time-lapsed microscopy was confirmed by the use of a control bscAb that did not bind to EphA2-positive cells (Fig. 4A) and in the absence of either bscEphA2xCD3 or of T cells (data not shown).

Analogous experiments were then done with bscEphA2xCD3 and the nontransformed epithelial cell line MCF10A, which expresses levels of cell surface EphA2 receptors that are similar to MDA-MB-231 cells (18). Subconfluent monolayers of MCF10A cells were used since the concept of epitope exclusion predicts that unobstructed EphA2 epitopes on the exposed (outer) edge of the monolayer should be available to bind bscEphA2xCD3, whereas cells in contact with each other should not. Cytotoxic T-cell activity against the MCF10A monolayer was only noted at the exposed edge; central parts of the MCF10A monolayer were largely unaffected by T cells except where stable linkages with adjacent cells failed and exposed previously inaccessible EphA2 binding sites (Fig. 4C). Over time, this front of cytotoxic T-cell activity (see arrows) eventually moved all across the monolayer suggesting sequential lysis at the exposed edge.

To determine if newly exposed EphA2 epitopes could bind bscEphA2xCD3, we disrupted a small portion of an intact MCF10A monolayer. Redirected T-cell lysis was largely confined to the outer

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**Figure 3.** Characterization of bscEphA2xCD3 in vitro cytotoxicity. A, kinetics of redirected lysis. CD3+ T cells were incubated with SW480 cells in the presence of serial dilutions of bscEphA2xCD3 for 4 h (■), 18 h (▲), 24 h (▼), or 42 h (●) at 37°C. Points, mean; bars, SD. EC50 values were estimated from the curves and are listed. B, influence of effector to target ratio on redirected lysis. CD3+ T cells were incubated with SW480 cells at a ratio of 20:1 (●), 10:1 (▲), 5:1 (▼), 1:1 (○), 1:2 (▲), or 1:5 (■) in the presence of serial dilutions of bscEphA2xCD3 for 42 h at 37°C. Points, mean; bars, SD. EC50 values were estimated from the curves and are listed. C, effects of available EphA2 receptor binding sites on the potency of redirected lysis. The estimated number of EphA2 molecules per cell for each tumor line (HeyA8, SW480, A549 human carcinoma lines, and M14 and A375 melanoma lines) was plotted against the respective EC50 values of the percentage of target cells lysed from four separate experiments. P and r2 of the linear regression curve are listed on the graph. D, effects of EphA2 surface density on redirected lysis. CD3+ T cells were incubated with the cell lines HeyA8 (■: 107,000 EphA2 receptors per cell), M14 (▲: 2,400 EphA2 receptors per cell), and SKMEL-28 (○: EphA2 receptors were below limit of detection) in the presence of serial dilutions of bscEphA2xCD3 for 18 h at 37°C. Points, mean; bars, SD. EC50 values were estimated from the curves and are listed.
edges of the disrupted monolayer where EphA2 previously occluded by intercellular junctions were now available to bind bscEphA2xCD3 (Fig. 4C). Our observation that redirected T-cell lysis of the MCF10A cells located at the disrupted edges of the monolayer provided initial evidence against the idea that central cells may be more resistant to bscEphA2xCD3-mediated killing than those growing at the edge. To further explore this possibility, we used a bscAb that targets CD3 and a different molecule (EpCAM) on MCF10A cells by an epitope that is not subject to exclusion. When exposed to this particular EpCAM/CD3–specific bscAb, T cells were able to lyse MCF10A cells across the entire monolayer (Fig. 4B). Cytotoxic activity of the T cells was further increased where the monolayer was intentionally disrupted (Fig. 4B, disrupted monolayer), thus showing that even the central cells were sensitive to redirected lysis. To confirm the specificity of the cytotoxicity reactions, we used a control bscAb molecule that could associate with T cells but not MCF10A cells. This control bscAb did not affect the cell monolayer (Fig. 4A and B), and the T cells moved over the monolayer and remained randomly distributed on top of the MCF10A cells during the period of observation. Together, these studies provide further evidence that bscEphA2xCD3 exhibits the property of epitope exclusion and may provide a means for the selective targeting of malignant cells that express these EphA2 epitopes.

BscEphA2xCD3 is efficacious in vivo. To determine if the potent ex vivo activity of bscEphA2xCD3 would translate to inhibition of tumor growth in vivo, an adoptive transfer model was used. Immunodeficient NOD/SCID mice were engrafted with a s.c. mix of EphA2-positive human colorectal cancer SW480 cells and unstimulated human CD3+ T cells from healthy donors. At the beginning of the experiment, animals were then treated with five daily i.v. doses of bscEphA2xCD3. A bscAb that binds CD3 and another human-specific target (CD19) that is not expressed on the SW480 tumor cells was used as the control. Treatment with bscEphA2xCD3 significantly inhibited tumor growth in a dose-dependent manner (Fig. 5A), whereas the human CD19/

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<th>Figure 4. bscEphA2xCD3 mediates redirected lysis of tumor cells on the exposed edge of a nontransformed epithelial cell monolayer. Nontransformed MCF10A (A–C) and transformed A549 and MDA-MB-231 cells (D) were plated in 48-well plates for 24 h to establish monolayers. Stimulated CD8+ T cells (E/T ratio 1:1) were added with 100 ng/mL of a negative control bscAb that binds CD3 and does not bind to MCF10A cells (A), 100 ng/mL of a positive control EpCAM/CD3-specific bscAb (B), or 100 ng/mL of bscEphA2xCD3 (C and D). Immediately before addition of the T cells, the indicated monolayers were scraped with a pipette. Time-lapsed microscopy was recorded for 20 h with approximately one picture exposed per minute (see Supplementary Data). Images represent specific time intervals as indicated after the addition of T cells. “Intact monolayer” and “disrupted monolayer” represent two separate experiments. C, white arrows, edge of the MCF10A monolayer initially attacked and lysed by the CD8 T cells in the presence of bscEphA2xCD3. Dotted lines highlight the edge of the disrupted cell monolayer.</th>
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CD3–specific bscAb (nonrelevant control) had no such effect on SW480 tumor outgrowth. The control bscAb had no effect on tumor growth; similarly, the PBS vehicle or five 20-μg doses of bscEphA2xCD3 administered in the absence of T cells did not alter growth of tumor (Fig. 5B). Tumors grew equally well after engraftment of SW480 cells alone or after engraftment of SW480 cells mixed with T cells. Thus, in the absence of bscEphA2xCD3 treatment, T cells alone had no effect on tumor growth.

To determine if the presence of peripheral and lymphoid human T cells might absorb bscEphA2xCD3 and potentially reduce its antitumor efficacy, we used the xenograft model of SW480 cells combined with human T cells and added additional peripheral human T cells by i.v. administration. NOD/SCID mice were engrafted s.c. with a mix of SW480 tumor cells and human T cells as done above. In parallel and before the first bscAb treatment, mice were injected i.v. with unstimulated human CD3+ T cells, which in a separate experiment were shown to remain viable and circulating in the animals for at least 3 days (Fig. 5D). Notably, injection with excess human T cells in the periphery did not alter the antitumor efficacy of bscEphA2xCD3 (Fig. 5C).

Discussion
In this study, we report the development of a novel EphA2/CD3–targeting bscAb that stimulates T cells to selectively kill tumor cells that express accessible EphA2 epitopes. BscEphA2xCD3 is active in vitro and in vivo and thus provides a potent means of killing tumor cells. Different criteria reveal the high potency and selectivity of bscEphA2xCD3 to lyse targets cells at relatively low concentrations (picomolar, ng/mL) and against tumor cells that express a wide range of antigen densities. Additionally, tumor lysis can approach 100% and occur at low (1:1) E/T ratios. Given the

![Figure 5](http://www.aacrjournals.org/)

**Figure 5.** Dose-dependent effect of bscEphA2xCD3 on s.c. SW480 growth in NOD/SCID mice. A, effect of bscEphA2xCD3 on tumor growth. NOD/SCID mice (n = 6) engrafted s.c. with SW480 cells mixed with human PBMC enriched for CD3+ T cells and the indicated doses of target antigen/CD3–specific bscAb were administered i.v. on study days 0 to 4 (arrows). *, P < 0.01; #, P < 0.05 (unpaired Student’s t test). B, negative control treatment groups. NOD/SCID mice (n = 6) were inoculated s.c. with SW480 cells in the absence (SW480 only and bscEphA2xCD3 only) or presence of human CD3+ T cells. PBS vehicle control (PBS), 20 μg of a bscAb that binds CD3 and is nonreactive to SW480 cells, or 20 μg of bscEphA2xCD3 was administered by tail vein injections on study days 0 to 4 (arrows). C, effects of the addition of peripheral human T cells on in vivo responses to bscEphA2xCD3. NOD/SCID mice (n = 6) were engrafted s.c. with SW480 cells mixed with human PBMC enriched for CD3+ T cells and where indicated, an additional i.v. engraftment of human PBMC enriched for CD3+ T cells from the same donor. Nonrelevant bscAb or bscEphA2xCD3 was administered i.v. on study days 0 to 4 (arrows). The significance of the PBS- and bscEphA2xCD3-treated groups that received additional intravenous CD3+ T cells was compared by a paired Student’s t test. *, P < 0.01; #, P < 0.05. D, time course for depletion of viable human CD3+ T cells in mice after i.v. engraftment. SCID mice that were treated i.v. with anti-asialo GM1 antibody were engrafted by i.v. injection with human CD3+ T cells. Spleens were examined by flow cytometry at the indicated time points after engraftment for the presence of viable, human CD3+ T cells that excluded propidium iodide. Percentage of viable human CD3+ T cells was compared with the total number of live and dead cells contained within the mouse spleen. The half-life (t1/2) of human T cells in the mice was estimated using a one-phase exponential decay, nonlinear regression analysis. Note: Previous studies suggest that after s.c. administration, human T cells are viable for at least 8 d when combined with human tumor cells (32, 34, 37).
highly potent tumoricidal effects of diverse bsAbs of the BiTE class (27), we designed a new EphA2/CD3–specific bsAb from an EphA2-specific antibody, which recognizes an epitope that is preferentially exposed on transformed cells (18). Altogether, the potency and selectivity of bsEphA2xCD3 suggest that there is an opportunity to develop a highly potent, but well-tolerated, new therapeutic strategy for the treatment of the many cancers that overexpress EphA2.

The past decade has shown that chimeric and humanized mAbs provide a means of selectively targeting malignant cells while sparing toxicities to normal tissues (20). A growing number of these mAb-based cancer therapeutics is available and efficacious (20) however, the efficacy of mAb therapy for cancer treatment remains limited (20), which prompted the search for and development of new antibody-based technologies with improved antitumor efficacy (44, 45), including bsAbs of the BiTE class (27). Our present study illustrates certain potential advantages that BiTE molecules may convey relative to conventional antibody therapies. One possible advantage is that bsEphA2xCD3 and another BiTE (MT103/MEDI-538) show potent tumor cell killing in vitro and in the clinical setting (46), respectively, at concentrations that are magnitudes lower than have been associated with standard biologics-based therapies (21–23). Likewise, recent clinical information suggests that mAb therapies may encounter certain limitations that should not affect BiTE antitumor efficacy. For example, single nucleotide polymorphisms of FcγRIIIa may limit antibody-dependent cell-mediated cytotoxic activity of naked mAbs (24–26); BiTE activity does not depend on Fcγ receptors for its activity (27). The relatively small size of bsAbs (55–60 kDa) may also facilitate better penetration into solid tumors (47). Although these features are promising, further analyses will be necessary to fully understand the differences in functional properties of BiTE molecule compared with other active or passive immune strategies.

EphA2/CD3–specific bsAbs may also have advantages relative to active immunotherapy for EphA2-expressing cancer. For example, innate tolerance to certain antigens has limited the usefulness of tumor vaccine approaches (48). In contrast, BiTE molecules may be insensitive to disease-associated desensitization of T cells that recognize tumor (27, 49). Tumors often display alterations in HLA expression or antigen processing and presentation, which can limit the effectiveness of active immunotherapy (50). BiTE molecules are unlikely to be affected by such tumor resistance mechanisms (28).

Tumor vaccines may need to mount a robust immune response to overcome tolerance. However, a potent tumor vaccine may also elicit autoimmune or inflammatory responses to normal tissues that express the antigen, or which are subject to epitope spreading, which could limit the clinical application of active immunotherapy (48). BiTE molecules are unlikely to show such effects, and, indeed, they may provide opportunities to combine the specificity of antibody-based therapies with the potent killing power of T lymphocytes. A further potential advantage of the BiTE approach is that it could make use of tumor-infiltrating lymphocytes that frequently are observed within the solid tumor microenvironment (35, 49). EphA2/CD3–specific bsAbs are designed to recruit these T cells and redirect their activity in a tumoricidal manner.

In summary, our present findings suggest opportunities for using an EphA2/CD3–directed bsAb of the BiTE class in cancer therapy. The overexpression of EphA2 in certain types of solid tumors and its relatively low expression in normal tissue may be exploited to develop therapies that maximize efficacy while minimizing toxicity to normal tissues.

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