Increasing Anticarcinoma Activity of an Anti-erbB2 Recombinant Immunotoxin by the Addition of an Anti-EpCAM sFv

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Abstract

Purpose: erbB2, the product of the Her2-neu gene, is a well-established therapeutic target for antibody-based biologicals, but anti-erbB2 antibody-toxin fusion proteins are limited in their activity. The goal of this study was to determine if genetically adding an sFv targeting epithelial cell adhesion molecule (EpCAM) to an anti-Her2 sFv immunotoxin would result in enhanced anticancer activity.

Experimental Design: In vitro studies were done in which the new bispecific immunotoxin DTEpCAM23 was compared with monospecific immunotoxins (DTepCAM and DT23) to quantitate immunotoxin activity. Mixtures of monospecific immunotoxins were tested to determine if they were as effective as the bispecific immunotoxin. Binding and internalization studies were also done. In vivo, bispecific immunotoxins were given i.t. to athymic nude mice bearing HT-29 human colon cancer flank tumors and i.p. to mice with i.p. tumors.

Results: DTepCAM23 bispecific immunotoxins showed far greater activity than monospecific immunotoxin (sometimes over 2,000-fold) against most tumor lines. Bispecific immunotoxin was superior and selective in its activity against different carcinoma cell lines. Bispecific immunotoxin had greater activity than monospecific immunotoxin indicating an advantage of having both sFvs on the same single-chain molecule. Binding and internalization studies did not explain the differences between bispecific immunotoxin and monospecific immunotoxin activity. Orientation of the sFvs on the molecule had a significant effect on in vitro and in vivo properties. The bispecific immunotoxins were more effective than the monospecific immunotoxin in the flank tumor mouse model.

Conclusions: The synthesis of bispecific immunotoxin created a new biological agent with superior in vitro and in vivo activity (over monospecific immunotoxin), more broad reactivity, more efficacy against tumors in vivo, and diminished toxic effects in mice.

The 185-kDa transmembrane receptor product of the Her-2 oncogene (erbB2) has been widely studied as a carcinoma cell marker (1). Clinical research has shown erbB2 to be overexpressed on 30% to 40% of breast and ovarian cancers (2), and high levels of expression are linked to poor patient survival and prognosis (3). Increased expression of erbB2 is also observed in colon, prostate, lung, and gastric cancers (4–7), making it a popular therapeutic target for carcinomas. Current therapies directed at patients with high Her-2 expression levels, such as the monoclonal antibody trastuzumab (Herceptin) and small molecule inhibitor gefitinib (Iressa), have shown promising results (8, 9), but treatment resistance is still a problem.

As a solution, erbB2 has been targeted with immunotoxins, constructed by linking an anti-erbB2 sFv to catalytic toxins, such as Pseudomonas exotoxin and diphtheria toxin (DT), to specifically kill target cells (10). sFvs are single-chain molecules created by linking the variable regions of both the light and heavy chains of antibodies and are the smallest fragments of antibody (about 20 kDa) that will still bind antigen. Previously reported anti-erbB2 immunotoxins have shown promise in clinical studies (11, 12); however, other erbB2-targeting immunotoxins have resulted in high levels of toxicity when administered to patients (13).

To improve immunotoxin targeting of erbB2, we developed a bispecific immunotoxin by adding a different sFv targeting epithelial cell adhesion molecule (EpCAM) to the same single-chain recombinant anti-erbB2 immunotoxin molecule. This original erbB2-targeting immunotoxin (DT23) was made by splicing the gene encoding the anti-erbB2 sFv (e23) to DT390 (14). Other studies using a B-cell lymphoma model have shown that anticancer efficacy can be increased by adding an anti-CD19sFv to an anti-CD22 DT390 immunotoxin fusion protein (15).

EpCAM, a 40-kDa transmembrane glycoprotein encoded by the GA733-2 gene located on chromosome 4 (16), was identified as a specific marker of human colorectal carcinomas.
(17). Subsequent research showed that EpCAM was present in high numbers on a variety of human epithelial tumors compared with normal human tissue (18). EpCAM was selected because it has been used as a target for immunotoxin in past studies (19, 20).

DT is an established molecule for immunotoxin construction due to its irreversible catalytic activity and previous research showing that introduction of a single molecule into the cytosol is sufficient to kill the cell (21). Recombinant DT390 is a truncated form of DT devoid of portions of the native binding domain. DT390 was chosen for this study because previous research revealed a series of internal frame deletion mutations that established amino acid 389 as the ideal location for genetic fusion of DT and desired binding molecules (22).

In this study, we report that the addition of an anti-EpCAM sFv to an erbB2-targeting immunotoxin markedly improved in vivo killing of erbB2 targets regardless of the amount of erbB2 expression. One advantage of recombinant fusion proteins is that they can be genetically altered. Reversing the orientation of the EpCAM sFvs on DT23EpCAM, thus creating DTEpCAM23, decreased in vivo toxicity and improved its antitumor activity in vivo against erbB2+EpCAM+ targets in two aggressive nude mouse xenograft models.

Materials and Methods

Construction of DTEpCAM23. Synthesis and assembly of hybrid genes encoding the single-chain bispecific immunotoxin DTEpCAM23 was accomplished using DNA shuffling and DNA ligation techniques. Illustrations of the immunotoxins featured in this research are shown in Fig. 1A. The fully assembled genes (from 5′-end to 3′-end) consisted of an NcoI restriction site, an ATG initiation codon, the first 389 amino acid of the DT molecule (DT390; ref. 23), the VH and VL regions of humanized anti-EpCAM (MOC31; ref. 24) and anti-HER2 (e23; ref. 14) linked by a 20-amino-acid segment of human muscle aldolase (PSGQAGAAASELSFVSNHAY), and a XhoI restriction site. A second gene encoding DT23EpCAM was constructed that differed only in the orientation of the sFv regions. For each molecule, the upstream (5′) gene segment is listed first in the name of each respective molecule. The resultant 2,725-bp NcoI/XhoI fragment gene was spliced into the pET21d expression vector under control of an isopropyl-β-D-galactopyranoside–inducible T7 promoter (Fig. 1B). DNA sequencing analysis (Biomedical Genomics Center; University of Minnesota) was used to verify that the gene was correct in sequence and had been cloned in frame. Genes for monospecific immunotoxins splicing DT390 to anti-EpCAM sFv (DTEpCAM) and anti-erbB2 sFv (DT23) were created using the same techniques.

Two additional bivalent fusion proteins containing the DT390 fragment were created as specificity controls. BIC3 was produced by joining two repeating sFvs specific for human CD3ε to DT390. Anti-CD3ε binds to a domain of the T-cell receptor (25). Also used in this study was a bispecific immunotoxin consisting of duplicate anti-CD22 sFvs linked to DT390 (DT2222). CD22 is a human B lymphocyte–specific glycoprotein that shows increased expression in the majority of B-cell leukemias and lymphomas (15).

Protein expression, refolding, and purification. All immunotoxins used in this study were expressed, refolded, and purified as previously reported (15). Following purification, all immunotoxins were of the appropriate molecular weight and >90% pure when analyzed by SDS-PAGE (Fig. 1C). Large-scale preparations yielded ∼3 to 5 mg of purified protein per liter of bacterial culture.

Cell culture. The following cell lines were obtained from the American Type Culture Collection: BT-474, MCF-7, SK-BR-3, SK-OV3, LS-174T, HCT116, HT-29, Calu-3, DU-145, PC-3, A431NS, U-87MG, U-373 MG, Daudi, and C26. Table 1 describes the species and tissue of origin for all cell lines. All carcinoma and glialoblastoma cell lines were grown as monolayers in tissue culture flasks, and Daudi cells were grown in suspension. Cells were maintained in either RPMI 1640 (HT-29, SK-BR-3, BT-474, LS-174T, HCT116, Calu-3, DU-145, Daudi, and...
Healthcare) was added (1
A
Assays measuring 3H-leucine incorporation were done with leucine-free
overnight at 37
in a 96-well flat-bottomed plate in appropriate media and incubated
of either bispecific immunotoxin, and plates were incubated in 37
Each well was aspirated and replaced with 1 mL of 100 nmol/L solution
well) were plated in a 24-well plate. The following day, medium from
9.0)]. Labeled protein was separated from unbound FITC using NAP-5
DT23EpCAM were FITC-labeled at a 1:24 ratio (protein/FITC) for
(FACS) –based saturation binding assay (26), DTEpCAM23 and
target cells, using a previously reported fluorescence-activated cell sorting
software (BD Biosciences). V-FITC binding was analyzed on a FACSCalibur with Cell Quest
Biosciences) as described in the manufacturer's directions. Annexin
with 250
When cells were 80% to 90% confluent, they were passaged using
C26) or DMEM (SK-OV-3, A431NS, U-87MG, and U-373MG) supple-
C atmosphere containing 5% CO2. In addition to the preceding
1000 µg/mL streptomycin. In addition to the preceding
insulin. Cell cultures
C26) or DMEM (SK-OV-3, A431NS, U-87MG, and U-373MG) supple-
C were centrifuged and resuspended in trypan blue
j
C were incubated with 106 HT-29 or SK-BR-3 cells in a 100
size exclusion column (GE Healthcare). FITC-labeled immunotoxin were incubated with 108 HT-29 or SK-BR-3 cells in a 100 µL volume of FACS buffer (PBS + 2% fetal bovine serum) for 45 min. Following three washes with 500 µL of FACS buffer, cells were analyzed using FACS Calibur. To determine the dissociation constant (Kd), the maximum number of binding sites (B max), mean fluorescence intensity (MFI) was plotted versus immunotoxin concentration and analyzed with Prism software (GraphPad Software).

FACS-based internalization assays were also done to compare the
activity of different bispecific and monospecific immunotoxins on various human carcinoma cell lines
Table 1. Activity of different bispecific and monospecific immunotoxins on various human carcinoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>IC50 (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTEpCAMe23</td>
<td>DTe23EpCAM</td>
<td>DTe23</td>
</tr>
<tr>
<td>BT-474</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Human, breast</td>
<td>0.045</td>
</tr>
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<td>SK-BR-3</td>
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<td>0.0032</td>
</tr>
<tr>
<td>SK-OV3.ip1</td>
<td>Human, ovarian</td>
<td>0.031</td>
</tr>
<tr>
<td>LS-174T</td>
<td>Human, colon</td>
<td>0.0058</td>
</tr>
<tr>
<td>HCT-116</td>
<td>Human, colon</td>
<td>0.035</td>
</tr>
<tr>
<td>HT-29</td>
<td>Human, colon</td>
<td>0.0018</td>
</tr>
<tr>
<td>Calu-3</td>
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<td>0.02</td>
</tr>
<tr>
<td>DU-145</td>
<td>Human, prostate</td>
<td>0.19</td>
</tr>
<tr>
<td>PC-3*</td>
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<td>0.026</td>
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<tr>
<td>A431NS</td>
<td>Human, squamous</td>
<td>2.1</td>
</tr>
<tr>
<td>U373</td>
<td>Human, glioblastoma</td>
<td>&gt;100</td>
</tr>
<tr>
<td>U87</td>
<td>Human, glioblastoma</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Daudi</td>
<td>Human, lymphoma</td>
<td>&gt;100</td>
</tr>
<tr>
<td>C26</td>
<td>Mouse, colon</td>
<td>&gt;100</td>
</tr>
</tbody>
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*Proliferation assay conducted with 3H-leucine instead of 3H-thymidine.

Bioassay to measure cell proliferation. To determine the effect of immunotoxin on various cancer cell lines, proliferation assays measuring
3H-thymidine incorporation were used. Cells (105 per well) were plated out in a 96-well flat-bottomed plate in appropriate media and incubated
overnight at 37°C with 5% CO2. Immunosorin in varying concentrations was added in triplicate to wells containing cells. Incubation at 37°C and 5% CO2 continued for 72 h. [Methyl-3H]-thymidine (GE Healthcare) was added (1 µCi per well) for the final 8 h of incubation. Assays measuring 3H-thymidine incorporation were done using leucine-free media and a 24-h incubation with 3H-leucine. Plates were frozen to
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internalization efficiency of DTEpCAM23 and DTEpCAM. LS-174T cells
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were used for experiments.

Binding affinity and internalization of FITC-labeled immunotoxins. To quantitatively compare the binding of each immunotoxin to target
cells, using a previously reported fluorescence-activated cell sorting
(FACS) –based saturation binding assay (26), DTEpCAM23 and
DT23EpCAM were FITC-labeled at a 1:24 ratio (protein/FITC) for 3 h in a 50 mmol/L borate buffer solution [50 mmol/L boric acid (pH
9.0)]. Labeled protein was separated from unbound FITC using NAP-5
the University of Minnesota Institutional Animal Care and Use Committee. All animals were housed in microisolator cages to minimize the potential of contaminating virus transmission.

For flank tumor studies, mice were injected in the left flank with \(4 \times 10^6\) HT-29 cells suspended in 100 \(\mu\)L sterile PBS. Beginning on day 6 after tumor cell injection, mice were treated with daily i.t. injections of immunotoxins. DT23, DTEpCAM, and DT23EpCAM were given in alternating doses of 20 and 10 \(\mu\)g per animal, whereas DTEpCAM23 was given in 40 and 20 \(\mu\)g doses. All treatments were given in a 100 \(\mu\)L volume of sterile PBS. Tumor size was measured, and volume was determined as a product of length, width, and height.

A peritoneal metastasis model was developed by i.p. injecting nude mice with 1 mL solution of sterile PBS containing \(1 \times 10^6\) HT-29 cells. Treatment was initiated 1 day after tumor cell injection. Bispecific immunotoxin were administered i.p. in a 1 mL volume of PBS. All immunotoxins were given on daily on days 1 to 3, and subsequent injections were given as often as weight-monitored toxicity allowed through day 55. Animal weights and survival were monitored, and mice were euthanized if they lost >20% of body weight (28), became irreversibly jaundiced, or appeared moribund.

Statistical analyses. Group-wise comparisons of continuous data were made by Student’s \(t\) test. A computer program for compiling life table and statistical analysis by the log-rank test was used to analyze survival data. \(P < 0.05\) was considered significant.

Results

Efficacy and specificity of DTEpCAM23 in vitro. Proliferation assays were used to test the in vitro activity of DTEpCAM23. All immunotoxins were first tested against the SK-BR-3 human breast cancer cell line that expresses high levels of erbB2 (29). Figure 2A shows that against SK-BR-3 cells, monospecific DT23, which targets the erbB2 receptor only, was able to kill SK-BR-3 cells but only at higher concentrations (IC\(_{50}\) = 7.9 nmol/L). DTEpCAM, targeting EpCAM only, exhibited an IC\(_{50}\) of 0.08 nmol/L. However, the bispecific immunotoxin DTEpCAM23 showed an IC\(_{50}\) of 0.0032 nmol/L, representing a 2,468-fold increase in activity compared with DT23. The other bispecific immunotoxin (DT23EpCAM) showed similar levels of activity compared with DTEpCAM23 (data not shown). BIC3, a bivalent immunotoxin targeting the CD3 T-cell receptor (30), was tested as a negative control and exhibited only mild inhibition of SK-BR-3 cells. We next tested DTEpCAM23 against the HT-29 human colon cancer cell line (Fig. 2B), which expresses medium levels of erbB2 (31) and high levels of EpCAM (32). Interestingly, whereas the erbB2-targeting DT23 was not able to affect proliferation of HT-29 cell, the bispecific DTEpCAM23 showed a 10-fold increase in activity over the monovalent DT23. To ensure the activity of DTEpCAM23 was specific, we tested its effects on the proliferation of the EpCAM and erbB2 Daudi lymphoma cell line. Figure 2C shows that DTEpCAM23 did not affect Daudi cells at any concentration, whereas DT2219ARL, a bispecific immunotoxin targeting the CD19 and CD22 B-cell markers, effectively killed Daudi cells. These data showed that combining an EpCAM targeting sFv with an erbB2 targeting sFv

![Fig. 2. Effect of DTEpCAM23 on erbB2-expressing human breast cancer cells. Proliferation of (A) SK-BR-3, (B) HT-29, and (C) Daudi cells cultured with immunotoxins was measured by uptake of tritiated thymidine. Data was expressed as a percentage of control counts, where control is untreated cells. Mean untreated cell counts were 96,415 ± 8,657, 78,718 ± 7,485, and 91,604 ± 8,610 for SK-BR-3, HT-29, and Daudi cells, respectively. BIC3, a T-cell-specific immunotoxin was included as a negative control.](www.aacrcjournals.org)
on a single-chain molecule increases the potency against multiple breast cancer cell lines compared with either monospecific immunotoxin.

Cytotoxicity of bispecific immunotoxin against a variety of cancer cell lines. DTEpCAM23, DT23EpCAM, DT23, and DTEpCAM were tested against a number of cancer cell lines of different origins (Table 1). Both bispecific immunotoxins were consistently more potent at inhibiting proliferation of human carcinoma cells than the monospecific immunotoxins. Against the ovarian cancer cell line SK-OV3.ip1, the DTEpCAM23 had at least a 580-fold greater increase in activity compared with either monospecific immunotoxin. Against the prostate cancer cell line PC-3, DTEpCAM23 had at least a 756-fold greater increase compared with either monospecific immunotoxin. In certain instances, DT23EpCAM was more active than DTEpCAM23. Against the A431NS human epidermoid line, bispecific immunotoxins showed minimal advantage over monospecific immunotoxin. Neither DTEpCAM23 nor DT23EpCAM had any effect on human glioblastoma and lymphoma cells that lack expression of the erbB2 and EpCAM target molecules. Proliferation inhibition assays also showed that the bispecific immunotoxin did not have any cytotoxic activity towards the C26 murine colon carcinoma line, showing species specificity of these molecules. Together, these results show that DTEpCAM23 and DT23EpCAM are able to selectively kill a wide variety of human carcinoma cell lines in vitro and are generally more active than the monospecific immunotoxin.

Cytotoxicity of DTEpCAM23 is dependent on the presence of the DT portion of molecule. EpCAM23 and 23EpCAM, bispecific sFv's devoid of DT390, were synthesized and their effect against HT-29 cells was tested. Figure 2D shows that even at 100 nmol/L neither of the bispecific sFv's had any effect on the proliferation of HT-29 cells, whereas cells incubated with DTEpCAM23 were effectively killed at picomolar concentrations. In other experiments (data not shown), even 1,000 nmol/L concentrations of EpCAM23 did not affect proliferation of target cells. Thus, the proliferation inhibition activity of DTEpCAM23 and DT23EpCAM is dependent on the presence of a DT390 molecule.

DTEpCAM23 causes apoptotic death of target cells. To ensure that the bispecific immunotoxins were killing and not merely halting proliferation of target cells, a study measuring apoptosis of target cells was conducted. The ability of both DTEpCAM23 and DT23EpCAM to induce apoptosis was tested by monitoring the binding of Annexin to HT-29 cells treated
with each bispecific immunotoxin. Figure 3A shows that levels of Annexin binding increases in a time-dependent manner. These data show that a significant population of target cells incubated with DTEpCAM23 and DT23EpCAM undergo DT-catalyzed apoptosis.

**Increased activity of DTEpCAM23 is due to their combination in a single molecule.** To determine if the increased activity of bispecific immunotoxin was due to the presence of the two different sFvs on the same single-chain molecule, proliferation assays were done comparing HT-29 cells treated with DTEpCAM23 with cells treated with a mixture of DT23 and DTEpCAM. This combination of monospecific immunotoxin provides a number of binding molecules equivalent to the binding molecules on single-chain DTEpCAM23. Figure 3B shows representative results from assays done with HT-29 and SK-BR-3 cells. In each instance, the mixture of DT23 and DTEpCAM showed no increased activity towards target cells compared with the monospecific immunotoxin. These data show that increased activity observed with DTEpCAM23 is due to the presence of the two different sFvs on a single-chain molecule.

**Analysis of binding and internalization of immunotoxins.** To determine whether the enhanced cytotoxicity of the bispecific immunotoxin could be explained by enhanced affinity, the affinity of each immunotoxin for target cells was measured against HT-29 cells (Fig. 4A) or SK-BR-3 cells (Fig. 4B). Monospecific DTEpCAM and bispecific DT23EpCAM showed similar high affinities for HT-29 cells that express low levels of erbB2 ($K_d$ of 83.24 and 91.54 nmol/L, respectively). DTEpCAM23 showed a decreased affinity with a $K_d$ of 427 nmol/L. DT23 did not show appreciable binding to HT-29 cells. DTEpCAM, DT23EpCAM, and DTEpCAM23 all had similar $B_{max}$ values indicating nearly equivalent binding capacities (receptors occupied). Affinity was then tested using a second cell line (SK-BR-3), which express high levels of both erbB2 and EpCAM. Monospecific DTEpCAM once again showed a greater affinity with a $K_d$ of 139.5 nmol/L. Bispecific DT23EpCAM and DTEpCAM23 had lower $K_d$ values (218 and 442 nmol/L, respectively) but significantly greater binding capacities. DT23 once again showed the lowest affinity. Together, these data show the increased cytotoxicity of the bispecific immunotoxin is not solely a function of increased binding affinity, and increased binding capacity for the bispecific immunotoxin is dependent on the expression of both target antigens on the cell surface.

The internalization efficiency of both bispecific immunotoxins was also measured using FITC-labeled agents. Figure 4C shows (a) that more DTEpCAM was internalized than DT23EpCAM, indicating that enhanced cytotoxicity of the bispecific cannot be explained by enhanced internalization. (b) DT23EpCAM and DTEpCAM23 are internalized into LS-174T cells at different levels. In affinity studies, DT23EpCAM showed greater binding affinity for two different cell lines. Regarding the internalization studies, a greater percentage of initially bound DTEpCAM23 was brought into LS-174T cells following a 90-min incubation at 37°C. Figure 4, combined with our cytotoxicity studies, shows that (a) the enhanced cytotoxicity of our bispecifics does not seem to be related to enhanced binding or internalization, and that (b) the orientation of the EpCAM and e23 sFvs on DT23EpCAM and DTEpCAM23 have different effects on the binding and internalization capabilities of each bispecific immunotoxin.

**Toxicity of DTEpCAM23 and DT23EpCAM.** Next, we tested the in vivo toxicity of the bispecific immunotoxin to determine their potential for systemic administration for in vivo therapy. C57Bl/6 mice were given 5 i.p. injections of 20 μg of either bispecific immunotoxin every other day. Figure 5A shows the average body weight of animals in each group. A significant difference ($P < 0.05$) in toxicity-related weight loss was observed, with mice receiving DTEpCAM23 losing an average of 7.5% of their body weight compared with a 20.9% loss in the DT23EpCAM group. Additional mice were then given two 40-μg doses of either DTEpCAM23 or DT23EpCAM and studied for histology. Kidneys taken from mice treated with DTEpCAM23 and DT23EpCAM both showed signs of renal toxicity (data not shown). The proximal and distal tubules exhibited high levels of degeneration, and there were high levels of edema throughout. Many of the glomeruli were inflamed when compared with control tissue. The kidney from the DTEpCAM23-treated mouse showed less degeneration of renal structure with more intact tubules and more viable glomeruli present when compared with the kidney of a DT23EpCAM-treated mouse.

![Fig. 4. Binding and internalization of bispecific immunotoxins.](image-url) Concentrations of FITC-labeled DT23EpCAM and DTEpCAM23 were incubated with (A) HT-29 and (B) SK-BR-3 cells for 45 min. Following FACS, the MFI was plotted versus immunotoxin concentration. C. Internalization efficiency of bound immunotoxin was determined by incubating cells with bound FITC-labeled DT23EpCAM and DTEpCAM23 at 37°C. Internalized protein was differentiated from surface bound by quenching extracellular FITC fluorescence with trypan blue. Columns, percentage of initially bound protein internalized.
mouse. Liver sections from each mouse exhibit high levels of fatty degeneration as well as hepatocyte destruction. The DTEpCAM23 mouse showed more healthy hepatocytes and lower levels of degeneration than the liver from the DT23EpCAM mouse. Based on both toxicity-related weight loss and histologic evidence, DTEpCAM23 exhibited decreased levels of in vivo toxicity compared with DT23EpCAM.

**Treatment of HT-29 flank tumors in nude mice.** The in vivo efficacy of DTEpCAM23 and DT23EpCAM were tested in a HT-29 nude mouse flank tumor model. Once the tumors were palpable (day 6), the mice were divided into groups and treated with i.t. injections of either DT23, DTEpCAM, DTEpCAM23, DT23EpCAM, or PBS. Weight loss was measured to monitor toxicity and dictate dose. The decreased toxicity of DTEpCAM23 permitted it to be administered at least twice the dose of DT23EpCAM. Figure 5B shows the average tumor size of the animals in each of the treatment groups. Both bispecific immunotoxins showed an increased antitumor effect compared with the monovalent DT23 and DTEpCAM. However, only treatment with DTEpCAM23 resulted in an average tumor volume significantly different ($P < 0.01$) from mice injected with PBS. Figure 5B (inset) shows the difference in tumor volume of a DTEpCAM23-treated mouse (top) and a PBS-treated mouse (bottom) on day 37. All mice treated with DT23 either died or had to be euthanized by day 24 due to treatment-related toxicity, despite DT23 being administered at the lowest dose of any of the immunotoxins in the study. Differences in toxicity were once again observed between the bispecific immunotoxins, with DT23EpCAM-treated mice losing significantly more weight than DTEpCAM23-treated animals despite being given at half the dose (data not shown), confirming the findings in normal mice shown in Fig. 5A.

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**Fig. 5.** A, toxicity of bispecific immunotoxins on mice. C57Bl/6 mice ($n = 3$ per group) were injected i.p. every other day (five total injections) with 20 μg of either DTEpCAM23 and DT23EpCAM. Toxicity was monitored by animal weight loss. Points, mean weights of three animals in each group; bars, ±1 SD. Arrows, days of immunotoxin injection. The curves are significantly different ($P < 0.05$), B, treatment of HT-29 flank tumors with DTEpCAM23. Antitumor effect of i.t. injections of PBS, DT23, DTEpCAM, DTEpCAM23, or DT23EpCAM on HT-29 flank tumors. Nude mice ($n = 5$ per group) were s.c. injected with $4 \times 10^5$ HT-29 cells, and treatment was initiated once tumors became palpable (day 6). Treatment was administered as toxicity allowed. Mean tumor volume was calculated by $W \times L \times H$ as measured by digital calipers. Inset, day 37 tumor from one of the mice injected with PBS (bottom mouse, injected right flank) compared with a day 37 mouse treated with DTEpCAM23 allegedly “cured” (top mouse, injected left flank). C, tumor volume of long-term survivors treated with DTEpCAM23. Immunotoxin injections stopped on day 38. Inset, H&E-stained section of HT-29 flank tumor from a mouse 24 h after immunotoxin injection with DT23EpCAM. White arrows point to areas of live tumor cells. Black stemmed arrows indicate necrotic region resulting from immunotoxin-mediated cell killing. Histology was done on a mouse that was treated identically but was not part of this experiment. D, effect of DTEpCAM23 on i.p. HT-29 tumors. A model of i.p. colon cancer metastases was established by injecting $1 \times 10^5$ HT-29 cells into the peritoneum of groups of nude mice ($n = 5$ per group). Treatment with DTEpCAM23 or DT2222 (negative control) began on day 1 and continued as toxicity allowed. Kaplan-Meier survival curve. The DTEpCAM23 and no-treatment curves are significantly different ($P < 0.002$).
Figure 5C shows the long-term antitumor effect on three of the mice in DTEpCAM23 group. These mice were healthy even 110 days after tumor implantation and were apparently “cured.” Figure 5C (inset) shows an H&E-stained section of HT-29 tumor that had been i.t. injected with DT23EpCAM. The white arrows point to areas of living tumor tissue that are highly vascularized and densely packed. The black stemmed arrows show necrotic areas specifically induced by the immunotoxin injection. Extracellular matrix is totally disrupted in this region, along with the destruction of all viable tumor tissue. Overall, these data show that local administration of DTEpCAM23 has significantly greater antitumor activity against HT-29 flank tumors than either monospecific immunotoxin or DT23EpCAM due to its increased activity and increased therapeutic window.

**Efficacy of DTEpCAM23 and DT23EpCAM against i.p. model of colon cancer.** To simulate peritoneal seeding of colon cancer cells following surgical tumor resection, 10^6 HT-29 cells were injected i.p. into nude mice. Mice were divided into groups given 20-μg i.p. injections of DTEpCAM23 (began on day 1), DT23EpCAM, DT2222 (irrelevant control), or no treatment. Figure 5D shows the Kaplan-Meier survival curve for the animals in the no-treatment, DTEpCAM23, and DT2222 groups. Mice treated with DTEpCAM23 lived significantly longer than untreated animals (124 versus 60 days), with three animals surviving the entire 150 days of the study (P < 0.002). DT23EpCAM treatment also increased the survival of animals with i.p. HT-29 tumors (116 days); however, there was only one survivor to 150 days (data not shown). Animals treated with DTEpCAM23 showed less weight loss and were injected more than twice as often as animals treated with DT23EpCAM (data not shown). Mice treated with DT2222 survived an average of 75 days, and the single surviving mouse in that group never showed signs of established tumor growth. This study shows treatment with DTEpCAM23 increased the survival of mice with i.p. HT-29 tumors compared with animals receiving either no treatment or an irrelevant treatment. Both animal models showed the increased efficacy and lower levels of toxicity with DTEpCAM23 compared with DT23EpCAM.

**Discussion**

The original contribution of this work is the discovery that cloning an anti-EpCAM sFv into a monovalent anti-erbB2 immunotoxin results in a bispecific immunotoxin with much greater activity than the monospecific immunotoxin. Further genetic manipulation of the DT23EpCAM construct by reversing orientation of the sFvs resulted in a protein with reduced toxicity and greater efficacy in vivo. Others have shown that immunotoxin activity is related to binding ability (33). The superior activity of the bispecific immunotoxin was not related to binding (a) because affinity studies revealed no real difference between monospecific DTEpCAM binding and bispecific immunotoxin binding. (b) Studies with a mixture of monospecific DTEpCAM and DT23 did not show additive killing. Bispecific immunotoxin enhanced activity against a variety of carcinomas (breast, colon, and lung) sometimes several thousand-fold but had no effect against lymphoid cancer or glioblastoma. Importantly, in vitro results correlated with in vivo findings and localized delivery of DTEpCAM23 resulted in the cure of some mice given aggressive HT-29 tumors.

An important observation of these studies is that we targeted and compared two markers that are known be present on carcinomas and known to be internalized when bound by immunotoxins (34, 35). The comparison of the activity of the monospecific immunotoxins DTEpCAM and DT23 revealed that EpCAM targeting was superior to erbB2 targeting in killing carcinoma cells. The addition of the anti-EpCAM sFv to the single-chain DT23 molecule created an entirely different profile. Despite the level of expression of erbB2, the bispecific immunotoxin was always more active against the carcinomas. Binding and internalization studies did not reveal an advantage for either bispecific immunotoxin over DTEpCAM. These data showed that the increased cytotoxicity of DTEpCAM23 and DT23EpCAM are not solely due to binding affinity and internalization efficiency. Studies have indicated that a second step (i.e., internalization) is a prerequisite to optimal immunotoxin activity (36). Despite the fact that portions of the translocation-enhancing region of the toxin are included in the DT390 cassette, this still does not guarantee that all immunotoxin will be able to enter and kill the cell after binding (37). Perhaps, simultaneous binding to EpCAM and erbB2 alters the endosomal trafficking of DTEpCAM23 within the tumor target cell, resulting in increased cytotoxicity.

Other immunotoxins targeting dual receptors have been reported. The sFvs of the single-chain fusion protein DT2219 selectively and simultaneously target human CD22 and CD19 on B cells, and this is effective in the therapy of systemic B-cell malignancy in a scid/hu mouse model (15). As in the case of DTEpCAM23 for human carcinoma, the bispecific immunotoxin DT2219 for human B-cell leukemia was much more effective than the corresponding monospecific immunotoxin. Interestingly, this was not the case for the anti-glioblastoma bispecific immunotoxin DTAT13 (38). This fusion protein simultaneously targets the cytokine receptors urokinase-type plasminogen activator receptor and interleukin-13 receptor with the same DT390 casette used in the DTEpCAM23 studies. This bispecific molecule was made by molecularly attaching human interleukin-13 to the human urokinase ATF-DT390 gene. This did not enhance (nor hinder) the activity of the molecule against human MG U373 glioblastoma cells. Together, these data suggest that some but not all types of bispecific immunotoxins are subject to cotargeting whereby bispecific immunotoxin binding is followed by superior activity.

In this study, we employed two different xenograft models of human carcinoma. *In vivo* models provide a means to analyze the activity of immunotoxin in a more physiologically relevant setting. Growing human cancer cells as flank tumor is a well-developed procedure commonly used to test therapeutic agents. One advantage of flank tumors is that they are easily measured, allowing accurate and immediate assessment of treatment efficacy. In our study, we showed that i.t. injection of DTEpCAM23 was able to significantly reduce the volume of established HT-29 flank tumors in nude mice. This treatment also resulted in long-term elimination of any visible tumor in 3 of 5 (60%) treated animals. In the second xenograft model, bispecific immunotoxin was administered i.p. to mice with established peritoneal tumor. This allowed us to determine the ability of DTEpCAM23 to effectively bind and kill tumor cells in a less localized environment. We found that treatment with DTEpCAM23 was able to more than double the average survival time of mice injected i.p. with tumor compared with untreated mice.
mice and resulted in long-term survivors. I.t. administration of an erbB2-targeting immunotoxin has been tested clinically and resulted in complete tumor regression in 40% of treated patients (13). However, the majority of immunotoxins that have shown efficacy in a clinical setting have been able to show in vivo efficacy when administered systemically instead of locally as in these experiments. Pilot studies showed that DTεpCAM23 was able to slow the growth of flank xenografts when injected i.p. Further studies are required to optimize dosing and antitumor effect.

The high levels of erbB2 expression observed on a variety of carcinomas and the recent clinical success of the erbB2-directed monoclonal antibody Herceptin make the HER2 gene product a promising therapeutic target (39). EpCAM is a desirable therapeutic target due to numerous studies showing its role as a cell surface marker for a number of types of carcinoma. A recent clinical study of more than 4,000 primary human carcinoma samples revealed that high levels of EpCAM expression were present on more than 82% of the samples, and only 5% showed no expression (40). One clinical study has even shown that coexpression of high levels of both erbB2 and EpCAM is correlated with poor prognosis in breast cancer patients (41), suggesting that a drug targeting both these moieties would be highly beneficial to these high-risk patients. Simultaneous targeting of these two antigens with a bispecific immunotoxin yielded excellent results, with DTεpCAM23 not only affecting erbB2-overexpressing cancer cells but also against cell lines with low (HCT116) to medial (HT-29) levels of erbB2. Table 1 shows that both bispecific immunotoxins used in this study had IgG₅ values at least a log less than DTεpCAM, even on cells not affected by the erbB2-targeting DT23. Blocking data (not shown) showed that blocking either erbB2 or EpCAM alone inhibited a portion of the cytotoxicity of DTεpCAM23, and blocking both targets eliminated all effects of the bispecific immunotoxin. Taken together, these data provide proof that both targeting molecules on DTεpCAM23 play a role in the cytotoxicity of the agent.

The low binding affinity of DT23 to both cell lines, including erbB2-overexpressing SK-BR-3 cells, is likely a property of the e23 sFv in a monovalent conformation because other studies show that bivalent e23 forms have significantly greater binding affinities (35). In addition, because monoclonal antibodies directed at different epitopes of erbB2 show varying levels of internalization (42), other erbB2-targeting sFvs might increase the binding, internalization and ultimately cytotoxicity of bispecific immunotoxin.

One initial goal of this study was to create bispecific immunotoxin targeting the erbB2 receptor with lower systemic toxicity than the monospecific erbB2-directed DT23. DTεpCAM23 and DT23EpCAM were significantly less toxic than DT23. In fact, the mean tolerated doses of both bispecific immunotoxins are more than twice that of DT23 (data not shown). One potential explanation for this difference in toxicity is the disparity in molecular weights between the bispecific DTεpCAM23 and DT23EpCAM (97.7 kDa) and the monospecific DT23 (68.4 kDa). Due to the homeostatic role of kidneys in maintenance of body fluid composition, many small proteins are filtered in and can cause renal cell damage (43). Previous research has shown that immunotoxins with two sFvs can be less toxic than monovalent immunotoxins targeting the same antigen (44).

The success of Herceptin in treating malignant breast cancer has led to a number of studies to determine its mode of action (45). Whether Herceptin acts by antibody-dependent cell-mediated cytotoxicity, down-regulation of erbB2, or cell cycle modulation, it renders its significant antitumor effect by simply binding to Her2 molecules on the cell surface. However, the bispecific sFv devoid of DT230 does not inhibit carcinoma cells, an observation common to recombinant sFvs. This could be attributed to decreased binding efficiency, or due to the lack of any component of the Fc region that is common to conventional antibodies and Herceptin. The activity of DTεpCAM23 is dependent on the presence of DT23 and Annexin V studies revealed that apoptosis had been induced in some treated cells. This mechanism is common to other DT-based immunotoxins (46).

Although DTεpCAM23 and DT23EpCAM differ from one another only in the orientation of their sFvs, they did show different biological properties. Table 1 reveals that DT23EpCAM consistently showed slightly better cytotoxic activity towards target cells. Binding and internalization studies with FITC-labeled bispecific immunotoxin showed that whereas DT23EpCAM had better binding properties, DTεpCAM23 was more efficiently internalized into target cells. Research has shown that differences in binding are observed in bispecific diabodies based on the orientation of the binding domains (47). The other main difference between DTεpCAM23 and DT23EpCAM was the levels of in vivo toxicity observed in mice treated with the immunotoxin. Figure 5 shows that injection with DTεpCAM23 resulted in significantly less weight loss than injection with DT23EpCAM. Subsequent in vivo efficacy studies mirrored this observation, with DTεpCAM23 being tolerated at higher and more frequent doses. This apparent difference between the molecules is not due to their purity but may be a result of their refolded conformation.

Studying the toxicity of DTεpCAM23 and DT23EpCAM in mice can be problematic due to the fact that the bispecific immunotoxins do not bind to murine EpCAM or erbB2. Due to the expression of both EpCAM and erbB2 on some normal human tissues (48, 49), toxicity is a concern for clinical utilization of the agents. Immunohistochemistry studies should be able to establish the extent of reactivity of DTεpCAM23 and DT23EpCAM with normal human tissues.

In summary, our research shows for the first time that bispecific immunotoxins that are effective and highly selective against a wide range of carcinomas can be developed, providing proof that genetic manipulation can be used to address the problems with biological drugs. The fact that the in vivo efficacy of Her2 targeting can be improved by the addition of an sFv-targeting EpCAM opens possibilities for the development of other bispecific agents for targeting carcinoma. Although toxic side effects of the molecule could be an important issue, particularly when the drug is administered systemically rather than locally, new delivery modes, such as gene therapy, may help address this problem (50).

Acknowledgments

We thank Dr. Deborah Todhunter, David Kuroki, Andy Sicheneder, Liz Taras, and Dr. Donald J. Buchsbaum (University of Alabama, Birmingham) for their assistance and suggestions.
References


Increasing Anticarcinoma Activity of an Anti-erbB2 Recombinant Immunotoxin by the Addition of an Anti-EpCAM sFv

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