Diabodies Targeting Epithelial Membrane Protein 2 Reduce Tumorigenicity of Human Endometrial Cancer Cell Lines

Kaori Shimazaki,1 Eric J. Lepin,3 Bo Wei,4 Agnes K. Nagy,4 Catherine P. Coulam,4 Sergey Mareninov,4 Maoyong Fu,4 Anna M. Wu,3 James D. Marks,6 Jonathan Braun,1,3,4 Lynn K. Gordon,2,5 and Madhuri Wadehra4

Abstract Purpose: Endometrial cancer is the most common gynecologic malignancy. One promising biomarker is epithelial membrane protein 2 (EMP2), and its expression is an independent prognostic indicator for tumors with poor clinical outcome expression. The present study assesses the suitability of EMP2 as a therapeutic target. Experimental Design: Human monovalent anti-EMP2 antibody fragments were isolated from a human phage display library and engineered as bivalent antibody fragments (diabodies) with specificity and avidity to both EMP2 peptides and native cell-surface EMP2 protein. Diabodies were assessed using cell death and apoptosis assays. In addition, the efficacy of EMP2 diabodies on endometrial cancer tumors was determined using mouse xenograft models. Results: Treatment of human endometrial adenocarcinoma cell lines with anti-EMP2 diabodies induced significant cell death and caspase-3 cleavage in vitro. These responses correlated with cellular EMP2 expression and were augmented by progesterone, which physiologically induces EMP2 expression. In vivo, treatment of subcutaneous human xenografts of HEC-1A cell lines with anti-EMP2 diabodies suppressed tumor growth and induced cell death in the xenograft. Conclusions: These findings suggest that EMP2 may be a potential pharmacologic target for human endometrial cancer.

Endometrial cancer is the most common gynecologic malignancy. In the United States, the death rate from endometrial cancer has doubled in the last 20 years, and currently, a woman has approximately 3% chance of developing endometrial cancer during her lifetime (1, 2). Endometrial cancer is classified into two major subgroups based on histology, clinical behavior, and epidemiology. The more common type I is associated with estrogen predominance and premalignant endometrial hyperplasia (3, 4). Type II is mediated by nonhormonal risk factors and often has a high-grade or high-risk histology with an aggressive clinical course (3). Incidence of endometrial cancer generally increases with age, with 75% to 80% of new cases occurring in postmenopausal women (5).

Primary treatment for endometrial cancer is surgical removal of the tumor, but recurrence is common, and other therapeutic interventions (radiotherapy, chemotherapy, and endocrine therapy) benefit only a subset of patients (6, 7). Presently, there are few biomarkers that distinguish endometrial cancer at the premalignant stage, although emerging efforts are targeting molecules that underlie the process of tumorigenesis (8, 9). Similarly, there are currently no biomarkers that can be targeted for tumor suppression and elimination. Thus, new modalities for early detection and treatment of endometrial cancer at premalignant and malignant stages of disease are needed to improve management and prognosis.

One promising biomarker appears to be epithelial membrane protein 2 (EMP2). EMP2 expression is associated with endometrial neoplasia (10). In endometrial cancer, EMP2 is an independent prognostic indicator for tumors with poor clinical outcome. EMP2-positive tumors, compared with EMP2-negative tumors, had a significantly greater myometrial invasiveness, higher clinical state, recurrent or persistent disease following surgical excision, and earlier mortality. As EMP2 expression was independent of other known biomarkers such as the estrogen receptor and progesterone receptor (10), EMP2 represents a unique biomarker for patients who are not responsive to current hormone or chemotherapy. Moreover,
EMP2 expression levels positively correlate with the increasing premalignant potential of proliferative endometrium. That is, there is a gradation of endometrial EMP2 expression, with minimal expression in normal proliferative or quiescent premenopausal endometrium, and increasing expression in patients with disordered proliferative endometrium, endometrial hyperplasia, and endometrium carcinomas.

EMP2 is a member of the growth arrest-specific gene 3/ peripheral myelin protein 22 four-transmembrane protein family with distinctive biochemical and physiologic roles (11–13). In the endometrium, EMP2 expression is regulated by progesterone and required for successful blastocyst implantation (14, 15). EMP2 appears to regulate trafficking of various proteins and glycolipids by facilitating transfer of molecules from post-Golgi endosomal compartments to appropriate plasma membrane locations. Specifically, EMP2 is thought to facilitate the appropriate trafficking of select molecules into glycolipid-enriched lipid raft microdomains (12). Glycolipid-enriched microdomains are cholesterol-rich microdomains, which are often associated with chaperones, receptosomes, and protein complexes that are important for efficient signal transduction (16, 17). Moreover, glycolipid-enriched microdomains are involved in correct sorting of proteins from the Golgi apparatus to plasma membrane (18–20). In this respect, modulation of EMP2 expression levels or its location on the plasma membrane alters the surface repertoire of several classes of molecules including integrins, focal adhesion kinase, class I MHC molecules, and other immunoglobulin superfamily members such as CD54 and glycosylphosphatidylinositol-linked proteins (21–22).

The basic receptor cell biology of EMP2 (controlling surface display and signaling function) and its prognostic association with endometrial cancer and premalignant states suggested that EMP2 may be a pharmacologic target in endometrial cancer. In this study, we tested this idea by selecting human anti-EMP2 diabodies from single-chain Fv (scFv) antibody library (23) and engineering them as small bivalent antibody fragments (diabodies), which have unique practical applications because of their avidity, specificity, and small size (24). Recently, diabodies were found to undergo rapid tumor uptake and fast blood clearance in murine models, showing their potential for immunodiagnostics and immunotherapies for cancer (25). Here, we show that in vitro treatment of endometrial adenocarcinoma cells with anti-EMP2 diabodies dramatically decreased cell proliferation by up-regulating caspase-dependent apoptosis. Moreover, anti-EMP2 diabodies were effective in reducing tumor size and inducing cell death in human endometrial cancer xenografts. These data suggest that pharmacologic manipulation of EMP2 may be a candidate target for endometrial cancer therapy.

### Materials and Methods

**Cell lines.** The human endometrial adenocarcinoma cell line HEC-1A (HTB112; American Type Culture Collection), RL95-2 (CRL-1671; American Type Culture Collection), Ishikawa (gift of Dr. Mark Pegram, University of California at Los Angeles), and mouse embryonic fibroblast cell line NIH 3T3 (CRI-1658; American Type Culture Collection) were cultured in appropriate medium supplemented with 10% FCS at 37°C in a humidified 5% CO2 and passaged every 7 days. In addition to HEC-1A wild-type cells, HEC-1A sublines were prepared to increase EMP2 expression using expression plasmids for a human EMP2 (hEMP2)-GFP fusion protein and control GFP (21). These sublines were termed HEC-1A/OE and HEC-1A/V, respectively. EMP2 expression levels in each cell line were determined by Western blot analysis.

**Phage library selection.** Phage library selection was carried out as described previously (26). Briefly, 1012 to 1013 phage from the 8.2 member phagemid library were first predepleted with 100 μL streptavidin magnetic beads (Invitrogen) in 2% milk PBS for 1 h at room temperature. The predepleted phage library was then mixed with 10 μg biotin-conjugated 24-amino acid peptides corresponding to the extracellular loop of hEMP2 and mouse EMP2 (mEMP2; DIHDKNAKFYPVTREG-SYGGSCSKS and DLIHQQNNKLYLLQEGSYYGSCSKS, respectively; ref. 27) for 1 h at room temperature. One hundred microliters of 2% milk PBS preblocked streptavidin magnetic beads were added to the phage mixture and rotated for 15 min at room temperature. Beads were washed extensively with 0.1% PBS/Tween 20, 2% milk PBS, and finally with PBS and bound phage was then eluted with 1 mL of 100 mmol/L triethylamine, neutralized with 500 μL of 1 mol/L Tris-HCl (pH 7.4), and added to 10 mL exponentially growing Escherichia coli TG1. Culture was then plated on 150 mm culture plates with 2 × 10^5 μL/mL ampicillin and 2% glucose agar plates overnight at 37°C. The next day, colonies were scraped from the plates and used to amplify the phage for the second round of selection described above. A total of three selections were done before screening and characterization of the selected phage antibodies.

**Diabody construction and production.** Binding specificity of expressed scFv was analyzed by ELISA as described previously (see ELISA below for details; ref. 23). scFv clones with high reactivity were selected for the construction of diabodies. Several different scFv clones were characterized and confirmed by DNA fingerprinting (28, 29) and DNA sequencing (30). pHEN phagemids from selected phage were isolated using QIAprep Spin Miniprep Kit (Qiagen). scFv inserts were then digested and cloned into pSYN I vector in-frame with a c-Myc and 6 His tag at the COOH terminus. To convert scFv fragments into diabody, 15-amino acid linker (AGTGGTGGAGGCGGGTGTCAGGCGGGATGGGCCTCGTGGGCGGCGGATCG) of the scFv was shortened to 5-amino acid linker (AGTGGTGCGGATCG) using QuickChange site-directed mutagenesis kit (Stratagene; ref. 31). Deletion mutation was confirmed by DNA sequencing analysis.

Expression and purification of the selected diabodies were carried out using a modified protocol described by Marks and Bradbury (23). Single colonies were picked from the plate and inoculated into 1 L/ colony of 2 × 10^8 100 μg/mL ampicillin at 250 rpm at 37°C. When A600 reached 0.8 to 1.0, protein expression was induced by addition of 1 mmol/L IPTG. The culture was shaken at 120 rpm at 30°C for 4 h and spun at 7,000 rpm for 15 min at 4°C. Pellets were then resuspended in 20 mL periplasmic buffer [200 mmol/L Tris-HCl, 20% sucrose, 1 mmol/L EDTA (pH 7.5)], and 290,000 units lysozyme (Epicentre) was added to each mixture. The mixtures were incubated at room temperature for 1 h and centrifuged at 10,000 rpm for 10 min. Supernatant was collected and frozen at −80°C until further use.

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temperature for 5 min and spun at 7,000 rpm for 15 min at 4°C. The pellets were then resuspended with 20 mL of 40 mmol/L MgSO4 and left on the ice for 10 min. The samples were spun again, and the supernatants from this spin were combined with the first supernatants. The mixture was then filtered with 0.45 μm filters and dialyzed in dialysis buffer [300 mmol/L NaCl, 20 mmol/L HEPES (pH 8.0)] overnight at 4°C. Next morning, the samples were filtered again with 0.2 μm filters and run through 5 mL of the Ni-NTA column (Qiagen). The column was washed with 20 mL wash buffer [300 mmol/L NaCl, 20 mmol/L imidazole, 20 mmol/L HEPES, 0.05% Tween 20 (pH 8.0)] and bound diabodies were eluted with 5 mL elution buffer [300 mmol/L NaCl, 250 mmol/L imidazole, 20 mmol/L HEPES (pH 8.0)] and dialyzed in endotoxin-free PBS overnight at room temperature. Samples were filtered with 0.22 μm filters and stored at -20°C until their use. Purity of the preparation was determined by size-exclusion chromatography profile (fast protein liquid chromatography; Superdex 75; Amersham Pharmacia Biotech) as necessary.

For preparative analysis of the diabody, purified diabody preparations were run on 4% to 20% Tris-glycine gel (Invitrogen) and bands were visualized using GelCode Blue Stain Reagent (Pierce). Gels were scanned and the band intensities were analyzed using the Image J program (NIH).

ELISA. Biotinylated 24-amino acid peptide (10 μg/mL; see the Phage Library Selection above) was coated onto streptavidin-coated 96-well plates (Roche Applied Science) in PBS for 1 h at room temperature. Plates were then washed with PBS and blocked with 2% milk PBS for 2 h at 37°C. Expressed phage antibodies or diabodies were added to each well, incubated at room temperature for 1 h, and washed with 0.05% PBS/Tween 20 for 20 minutes. Bound antibodies or diabodies were detected with mouse anti-c-Myc (9E10) antibody (Calbiochem) followed by horseradish peroxidase-conjugated anti-mouse antibody (BD Bioscience Pharmingen) and TMB solution (eBioscience). Plates were read by microplate reader model 550 (Bio-Rad) at 450 nm.

Fluorescence-activated cell sorting analysis. Cells were detached from a flask with 2 mmol/L EDTA, spun at 1,000 rpm for 3 min, and resuspended with BD Cytofix/Cytoperm solution (BD Bioscience Pharmingen) to a final concentration of 1 × 10⁶/mL. Cells were washed with BD Perm/Wash buffer (BD Bioscience Pharmingen) followed by a 30 min incubation with BD Perm/Wash buffer containing 2% bovine serum albumin on ice. After spinning, 2,000 rpm, cells were resuspended with BD Perm/Wash buffer containing 1 μg purified monoclonal diabody in 96-well plates on ice for 1 h. Cells were then washed with 200 μL BD Perm/Wash buffer three times. Bound monoclonal diabodies were detected with mouse anti-c-Myc (9E10) antibody (Calbiochem) followed by R-phycocerythrin-conjugated anti-mouse secondary antibody (BD Bioscience Pharmingen).

Serum stability assay. Diabody preparations were diluted in 200 μL human or mouse serum to a final concentration of 5 μg/mL and plated in a 96-well plate. The plate was incubated at 37°C for 15 min, 24 h, 48 h, or 72 h. Samples were collected, and the diabody serum stability was determined via ELISA method described above.

Cellular cytosis. Cells (5 × 10⁴) were incubated in 96-well plates with 0 to 25 μg/mL diabody. At 0 and 24 h, cells were stained with toluidine blue and then lysed in 2% SDS (BioWhittaker). The number of cells at each time point was quantitated in triplicate by the absorbance at 595 nm, and the SE was calculated. Each experiment was repeated at least three times.

Cell death analysis. For cell death analysis, 5 × 10⁵ cells were incubated in 6-well plates in 10% FCS. Cells were incubated with 12.5 μg/mL diabody A10 (control), KS49, or KS83 for 72 h. For hormone treatment, progesterone was added to treatment medium at 25 μmol/L as described previously [15]. Briefly, RL35-2 cells at 60% to 70% confluence were washed in PBS and subsequently incubated with progesterone (Sigma) in treatment medium [DMEM/Ham’s F-12 supplemented with 0.5% charcoal-stripped FBS (Omega Scientific), 1% penicillin/streptomycin, and 1% l-glutamine]. Cell viability was determined by trypan blue exclusion.

To determine the rate of apoptosis, cells were stained with Annexin V (Becton Dickinson Biosciences) and 7-aminoactinomycin D or propidium iodide. Cells were harvested at 24 to 48 h after diabody treatment as indicated in the figure legends. The cells were incubated for 15 min on ice with Annexin V-Cy3 and Cy7-amineactinomycin D as per manufacturer’s instructions and analyzed on a flow cytometer (Becton Dickinson Biosciences).

Caspase-3 activity. Cells were incubated as above and harvested 48 h after diabody treatment. Samples were normalized based on intact and lysed by boiling for 5 min in Laemmli buffer [62.5 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 0.01% bromophenol blue, 2% ME]. The lysate was separated on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Pharmacia). Membranes were incubated with 10% milk in PBS containing 0.1% Tween 20. An anti-caspase-3 mouse monoclonal antibody, 2 ng/mL final concentration (BD Biosciences), or anti-β-actin (Sigma) was added and incubated for 1 h. The membrane was washed three times with PBS/Tween 20 and then incubated for 45 min with a horseradish peroxidase-labeled secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG; 1:2,000 dilution; Jackson ImmunoResearch Laboratories). Proteins were detected by chemiluminescence (Amersham Pharmacia).

Native tissue toxicity. Six- to 8-week-old female wild-type (C57BL/6) mice were obtained from JAX laboratories. Animals were inoculated intravenously with increasing concentrations (0.5-5 mg/kg) of A10 diabody control, anti-EMP2 diabodies (K83 and K49), or a vehicle control (sterile PBS). Three mice were used per group and injected twice a week. After 14 days, serum was collected, and mice were euthanized by cervical dislocation. Tissues (kidney, liver, spleen, lung, and skin) were collected and fixed in formalin. Samples were processed by the Tissue Procurement Laboratory at University of California at Los Angeles. Tissue in tissue was assessed using H&E and validated by a pathologist. Serum alanine aminotransferase and direct and total bilirubin were assessed by the University of California at Los Angeles Medical Center Clinical Laboratories.

Tumor xenografts and treatment. Four- to 6-week-old nude BALB/c female mice were obtained from Charles River Laboratories and maintained at the University of California at Los Angeles in accordance with institutional review board procedures. Animals were inoculated subcutaneously with 1 × 10⁶ HEC-1A/V or HEC-1A/OE cells into the right and left shoulder flanks, respectively. On day 13 (tumor diameter = 3 mm), tumors were measured biweekly with 1 mg/kg anti-EMP2 diabody 83, control diabody 80, or a vehicle control (sterile saline) for up to 3 weeks. Six mice were used per group. Tumors were measured every 3 to 4 days using vernier calipers, and tumor volumes were calculated by the formula: 6 × larger diameter × smaller diameter (32). At day 30, tumors were excised and fixed in formalin. Tumors were processed for H&E staining by the Tissue Procurement Laboratory at University of California at Los Angeles. In addition, some sections were stained for EMP2 expression as outlined below.

Immunohistochemistry. The expression of EMP2 in paraffin-fixed tissue has been described previously (10). Briefly, sections were processed for antigen retrieval by incubating slides at 95°C for 20 min in 0.1 mol/L citrate (pH 6.0). Sections were stained using primary hEMP2 antisera (1:400) or the corresponding preimmune control at the same dilution overnight at 4°C. The antibody signal was detected according to the manufacturer’s instructions using the Vectastain ABC kit (Vector Labs). EMP2 expression was visualized using diaminobenzidine. Nuclei were counterstained using hematoxylin.

Statistical analysis. For the ELISA analysis, groups were analyzed by two-tailed Student’s paired t test at a 95% confidence level. Differences in the in vitro antiproliferative and in vivo effects of diabodies were evaluated using Student’s unpaired t test at a 95% confidence level (GraphPad Prism version 3.0; GraphPad Software).
Results

Construction and expression of anti-EMP2 diabodies. Anti-EMP2 scFv were isolated using phage library expressing $8.2 \times 10^8$ variable scFv as described previously (26). EMP2-specific scFv were selected using 24-amino acid peptides that represent second extracellular domain of hEMP2 and mEMP2. Fourteen clones were identified by hEMP2 ELISA, and of these, three independent clones were found by sequence features. These three clones were constructed and produced as diabodies; all were positive by ELISA, and one (KS49) was positive by flow cytometry for native EMP2 binding (see below). In addition, 14 clones were identified by mEMP2 ELISA, and of these, three independent clones were found by sequence features. These three independent clones were constructed and produced as diabodies; all were positive by ELISA, and one (KS83) was positive by flow cytometry for native EMP2 binding. As negative controls, two random preselection scFv were chosen (A10 and B3); neither were positive by ELISA with hEMP2 or mEMP2 in either the scFv or diabody format.

For the present study, KS49 and KS83 were chosen as representative scFv for hEMP2 and mEMP2, respectively. Two random preselection scFv, A10 and B3, were used as negative control antibodies. To increase the avidity of the selected scFv, we created divalent diabodies by shortening the scFv linker region to 5 amino acids (31). Diabodies were expressed in TG1 E. coli and purified as published previously (see Materials and Methods).

SDS-PAGE and size-exclusion fast protein liquid chromatography analysis of purified anti-EMP2 diabodies. Analysis of purified diabody proteins by SDS-PAGE in a reducing condition showed a single band around 25 kDa, which corresponds to an appropriate size of scFv or diabody monomer (Fig. 1A; ref. 26). Size-exclusion chromatography also showed the formation of a dimer with a protein retention time at 20.23 min (average of two experiments), matching with the expected size of the diabody (Fig. 1B; ref. 26). Both data indicated >95% purity of the prepared diabody samples.

Antigen specificity of anti-EMP2 diabodies. Specificity and titer of selected diabodies were initially tested by ELISA using plates coated with hEMP2 or mEMP2 peptides. KS49, a diabody selected against hEMP2 peptide, showed significant binding to hEMP2, whereas binding to mEMP2 was below detection (Fig. 1C). Reciprocally, KS83, a diabody selected against mEMP2 peptide, showed high reactivity to mEMP2 peptide, whereas reactivity to hEMP2 peptide was below detection (Fig. 1D). Negative control diabodies A10 and B3 showed minimal reactivity to either hEMP2 or mEMP2 peptides. As shown in Fig. 1C and D, diabody titration analysis showed a dose-dependent binding of the KS49 and KS83 to the hEMP2 region.
and mEMP2 antigens, respectively. KS49 and KS83 efficiently bound to their appropriate antigen with EC_{50} (the antibody concentration at which 50% of maximum binding occurs) of 53.1 and 9.32 ng/mL, respectively. Using monovalent scFv products of these two antibodies, the EC_{50} for cognate EMP2 peptide was >2 μg/mL (data not shown). Thus, divalency contributed to the avidity of the two anti-EMP2 diabodies.

Binding activity of diabodies was further assessed by fluorescence-activated cell sorting analysis using human endometrial adenocarcinoma cell lines RL95-2, Ishikawa, and the murine fibroblast cell line NIH 3T3, all of which are known to express EMP2 (representative data shown in Fig. 2). Both KS49 and KS83 showed significant reactivity against all three cell lines regardless of the difference in host species. This species cross-reactivity may reflect the close homology between hEMP2 and mEMP2 second extracellular domains (50% sequence identity and 90% sequence similarity; see Materials and Methods). Control diabodies A10 and B3 showed minimal detection against all cell lines, confirming the specificity of the anti-EMP2 diabodies against EMP2 proteins.

Diabody stability in serum. One of the practical usages of diabodies is therapeutic targeting of cancer tumors (33). To assess the stability of anti-EMP2 diabodies in physiologic condition, 5 μg/mL diabodies were incubated in either human or mouse serum at 37°C for 15 min, 24 h, 48 h, and 72 h. The retained stability was measured using an ELISA. The binding activity of the diabody was maintained over the 72 h period in both human and mouse serum (data not shown). The specificity, which was detected using relevant and irrelevant peptide antigens, was also retained for the prolonged incubation period.

Antibodies to EMP2 inhibit cellular growth. To determine if selective targeting of EMP2 may be an effective therapy in endometrial cancer, the endometrial adenocarcinoma cell lines RL95-2, Ishikawa, and HEC-1A-WT were used. Cells were treated with KS49, KS83, or the control diabody A10 (Fig. 3). Compared with control diabody, anti-EMP2 diabodies induced cellular cytostasis within 24 h. When cells were incubated with a range of recombinant antibody from 0 to 25 μg/mL, the recombinant clones KS49 and KS83 had a dose-dependent, antiproliferative effect on the endometrial cell lines RL95-2 and Ishikawa (Fig. 3A and B). In contrast, diabodies against EMP2 exhibited small effects on HEC-1A-WT cells, which have been shown to bear little EMP2 on the plasma membrane (21). Previous studies have characterized HEC-1A/OE cells that overexpress EMP2 as a GFP fusion protein (14, 21). In these cells, EMP2 protein levels are increased approximately 4-fold.

Fig. 2. Cellular binding analysis of purified diabodies using flow cytometry. Cellular binding of purified diabodies (right) was tested on RL95-2, Ishikawa, and NIH 3T3 cells using flow cytometry. Three independent experiments were done with similar results. Representative graph. Bold line represents the isotype control; grey line represents the diabody-stained sample.
strikingly, diabodies KS83 and KS49 significantly inhibited growth of HEC-1A/OE cells by 55% and 21%, respectively, over cells treated with the control diabody A10 (Fig. 3D).

**Diabodies to EMP2 induce apoptosis.** To determine if the decrease in cell number was associated with an increase in cell death, cells were assessed for apoptosis using flow cytometry. Endometrial carcinoma cell lines were treated with 12.5 µg/mL KS49, KS83, or control A10 diabodies for 24 h. Apoptotic cells were detected with Annexin V and 7-aminoactinomycin D and analyzed by flow cytometry. Anti-EMP2 diabodies induced pronounced cell death in RL95-2 cells (Fig. 4A). Small effects were seen in HEC-1A-GFP cell lines, but cell death was enhanced by overexpression of EMP2 (HEC-1A/OE; Fig. 4B and C). Thus, anti-EMP2 diabodies specifically increased apoptosis in a manner associated with increased EMP2 surface expression.

**Synergistic effects of progesterone.** RL95-2 cells express functional progesterone receptor A and B receptors (34, 35). As progesterone increases EMP2 expression (15), we predicted that progesterone treatment may enhance the rate of anti-EMP2 diabody-mediated apoptosis in these cells. Cells were stained with Annexin V and propidium iodide and analyzed by flow cytometry. Dramatically, the combination of progesterone (P4) and KS49 or KS83 treatment increased the number of Annexin V, propidium iodide-positive cells by 16.5% and 19.3%, respectively, compared with cells treated with diabody alone (Fig. 5A).

To confirm that combination of progesterone and EMP2-specific diabodies increase cell death over diabody treatment alone, cells were analyzed by trypan blue exclusion 72 h after treatment (Fig. 5B). Progesterone and KS83 or KS49 significantly increased cell death over progesterone and control diabody A10 treatment (P < 0.01 and P < 0.04; Fig. 5B). Moreover, progesterone significantly increased KS83 diabody-mediated cell death by 19.1 ± 3% over KS83 treatment alone (P < 0.05). Although not significant, progesterone also increased KS49-mediated cell death by 8.1 ± 3% over KS49 treatment alone (P = 0.07).
The Annexin V and propidium iodide staining suggested that anti-EMP2 diabodies induced an apoptotic mode of cell death in RL95-2 cells. To validate this effect, cells were treated with 12.5 μg/mL of the EMP2-specific diabodies KS83 and KS49 or control diabody A10 in the presence or absence of progesterone for 24 to 36 h. EMP2 and active caspase-3 was measured in equivalent cell lysates by Western blot analysis (Fig. 5C). As expected, progesterone treatment augmented EMP2 expression by approximately 2.5-fold (Fig. 5C, left). Moreover, significant differences in cleaved caspase-3 was detected on addition of KS83 compared with the control diabody A10 (P < 0.05; Fig. 5C and D). Strikingly, higher levels of activated caspase-3 were detected on addition of progesterone and KS49 and KS83 compared with the control A10 (P < 0.05 and P < 0.01, respectively). These results suggest that progesterone and KS49 or KS83 act synergistically to induce apoptosis of endometrial cancer cells.

**In vivo tumor targeting.** To evaluate the preclinical efficacy of EMP2 therapy, the toxicity of two anti-EMP2 diabodies (KS49 and KS83) and a control diabody (A10) was assessed in wild-type C57BL/6 mice. KS49 and KS83 bind a shared epitope in mEMP2 and hEMP2 and are thus useful for assays for toxicity to normal tissues as well as therapeutic modeling in xenograft assays. To assess normal tissue toxicity, anti-EMP2 and control diabodies were parenterally administered twice a week (ranging up to 9 mg/kg) over 2 weeks in wild-type mice (C57BL/6). No changes were observed in animal weight or in serum metabolic analytes for liver function (Table 1). Gross and microscopic examination of tissues also showed no abnormalities (data not shown). Notably, this examination reflected an absence of toxicity in lung and skin, which express high levels of EMP2 (27, 36). Thus, in this limited analysis, no toxicity was detectable by anti-EMP2 diabody to normal tissues.

To evaluate the efficacy of anti-EMP2 diabodies in vivo, an endometrial cancer xenograft model was created. Tumors from HEC-1A/V and HEC-1A/OE cells were established in the shoulder flanks of female BALB/c nude mice. After detectable tumor formation (day 13), anti-EMP2 diabody KS83, control A10, or a vehicle control (sterile saline) were injected intratumor biweekly, and progression of tumor size was measured by calipers. By day 30, KS83 had profoundly inhibited tumor growth of both HEC-1A/V and HEC-1A/OE tumors (Fig. 6A).

Tumors were excised on day 30. Interestingly, in vivo, both HEC-1A/V and HEC-1A/OE tumors revealed high, comparable levels of EMP2 expression (Fig. 6B). In tumors from both cell types, high levels of EMP2 were observed within the cytoplasm as well as on the membrane. Dramatically, excised tumors...
revealed greater than 4-fold differences in tumor size between KS83 and A10 treatment in both HEC-1A/V and HEC-1A/OE cells (Fig. 5C and D). Within HEC-1A/V tumors, H&E staining revealed large areas of necrosis in tumors treated with KS83 but not with A10 (Fig. 6C). Necrosis was more pronounced in KS83-treated HEC-1A/V than HEC-1A/OE tumors, perhaps as the result of clearance by immune cells (Fig. 6D).

**Discussion**

In this study, we developed recombinant human anti-EMP2 diabodies using filamentous bacteriophage library methodology, and the efficacy of these diabodies on endometrial cancer cell lines was assessed with regard to growth, apoptosis, and xenograft tumor formation. Diabody avidity and specificity for EMP2 peptide and native protein were confirmed by ELISA and flow cytometry using multiple cell lines. Biologically, treatment of various human endometrial adenocarcinoma cell lines with these anti-EMP2 diabodies resulted in a significant increase in caspase-dependent apoptotic cell death in vitro and reduced tumor volume and viability in vivo. These data suggest that EMP2 is a targetable molecule for pharmacologic induction of apoptosis in endometrial cancer cell lines.

It was notable that a human immunoglobulin gene library permitted successful production of anti-hEMP2 antibodies. Although these antibodies in effect detected a self-antigen, they should not be construed to have native autoimmune specificity, because the combinatorial library permits the creation of...
VH/VL pairings that may not have been represented in the native clonal populations (23). Conversely, the direct yield of human immunoglobulin reagents with such biologic activity avoids the complexity of reengineering nonhuman epitopes while retaining antigen specificity and avidity in rodent-derived reagents (37).

Successful therapeutic targeting of antibodies depends on tissue penetration and uptake, rapid blood clearance, and serum stability. Small antibody fragments such as scFv have rapid tissue penetration and fast clearance from the circulation, but monovalent reagents are limited by low binding affinity and avidity (38–41). Accordingly, we engineered selected anti-EMP2 scFv fragments into bivalent diabodies, which are known to have an increased avidity and stability, by shortening the linker region of scFv between heavy-chain variable region (VH) and light-chain variable region (VL; refs. 42, 43). SDS-PAGE and size-exclusion fast protein liquid chromatography data confirmed successful diabody formation with >95% purity and >20-fold increase in binding activity compared with original scFv.

Fluorescence-activated cell sorting analysis of anti-hEMP2 and anti-mEMP2 diabodies showed similar binding activity to native surface EMP2. This was specific for EMP2, because it was dependent on levels of native or engineered EMP2 expression. Interestingly, whereas anti-hEMP2 and anti-mEMP2 diabodies were species specific for isolated peptides by ELISA, they showed cross-species reactivity for cell-surface hEMP2 and mEMP2. It should be noted that the selecting scFv fragments into bivalent diabodies, which are known to have an increased avidity and stability, by shortening the linker region of scFv between heavy-chain variable region (VH) and light-chain variable region (VL; refs. 42, 43). SDS-PAGE and size-exclusion fast protein liquid chromatography data confirmed successful diabody formation with >95% purity and >20-fold increase in binding activity compared with original scFv.

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Anti-EMP2 diabody treatment exhibited significant antiproliferative effects by increasing caspase-3-related apoptosis in multiple endometrial adenocarcinoma cell lines. These effects on cell growth inhibition and apoptosis correlated with EMP2 expression levels of independent cell lines, suggesting that binding of EMP2 induced apoptosis signaling. In support of this idea, progesterone induction of EMP2 expression increased diabody-mediated cell death in RL95-2 cells. Moreover, recent data have shown that intravaginal injection of anti-EMP2 diabody in the murine genital tract dramatically reduced EMP2 expression in native endometrial epithelium. It should be noted that EMP2 exists in a physical complex with FAK and certain integrin isoforms and promotes FAK-Src activation. Because divergent signaling pathways are induced by integrin ligation, it is conceivable that apoptosis may be favored in the absence of FAK (44–46).

To determine the preclinical applicability of targeting EMP2, toxicity experiments were initially done. It is known that the highest levels of EMP2 occur in the lung, skin, and female reproductive tract (27, 36). Importantly, anti-EMP2 diabody treatment exhibited minimal toxicity as measured by weight loss, liver function, and changes in histology when administered systemically over a 2-week time frame. Furthermore, the reduction in tumor volume for both HEC-1A/V and HEC-1A/OE cells with anti-EMP2 diabodies suggest that targeting EMP2 may be a successful treatment. Interestingly, HEC-1A/V cells, which express modest levels of EMP2 on the plasma membrane in culture, appear to have EMP2 expression levels that are comparable with HEC-1A/OE generated tumors in vivo. Consequently, HEC-1A/V cells responded significantly to anti-EMP2 diabody treatment. We predict that this may be the result of extrinsic factors (such as steroid hormones) that are known to increase EMP2 expression. Additional experiments will need to be done to validate this prediction.

Apoptosis can involve activation of diverse caspase isoforms depending on the death receptor-mediated and mitochondrial pathways of apoptosis induction (47). In this study, caspase-3 activation was assessed, because it is the downstream event of all of these pathways (47). We note that EMP2 is important for integrin expression and function and also modifies surface

**Table 1. Effect of parenteral diabody**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Starting weight (g)</th>
<th>Final weight (g)</th>
<th>Direct bilirubin (mg/dL)</th>
<th>Total bilirubin (mg/dL)</th>
<th>Alanine aminotransferase (units/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>19.9 ± 0.7</td>
<td>19.8 ± 0.4</td>
<td>0</td>
<td>0.2</td>
<td>45 ± 6</td>
</tr>
<tr>
<td>A10</td>
<td>18.7 ± 0.2</td>
<td>19.5 ± 1.7</td>
<td>0</td>
<td>0.2</td>
<td>36 ± 8</td>
</tr>
<tr>
<td>K83</td>
<td>18.9 ± 0.9</td>
<td>19.8 ± 0.9</td>
<td>0</td>
<td>0.2</td>
<td>44 ± 8</td>
</tr>
<tr>
<td>K49</td>
<td>19.9 ± 1.4</td>
<td>20.3 ± 0.1</td>
<td>0</td>
<td>0.2</td>
<td>37 ± 7</td>
</tr>
</tbody>
</table>

NOTE: Mice were injected i.v. with sterile saline, control diabody A10, or anti-EMP2 diabodies (K83 or K49) biweekly for 14 d. Three mice were used per group. Mouse weights were determined at the starting and final day; serum analytes were determined from blood obtained on the final day.


display of glycolipid-enriched microdomains and their associated membrane proteins. Accordingly, EMP2 may modulate integrin-dependent signaling associated with survival signaling or by other glycolipid-enriched microdomain-associated receptors. For example, K-ras and HER-2/neu have been identified as an endometrial cancer-associated oncogenes that stably interact with the plasma membrane and regulate activation of selective signaling pathways via lateral diffusion and interaction with

Fig. 6. Anti-EMP2 diabodies reduce tumor load in vivo. A, HEC-1A/V or HEC-1A/OE cells were injected subcutaneously into nude BALB/c female mice (left). At day 13 (arrow), mice were injected twice a week with 1 mg/kg anti-EMP2 diabody 83, control diabody A10, or sterile saline. Tumor volume was calculated using calipers \((n = 6)\). B, immunohistochemistry was used to analyze EMP2 expression in untreated tumors. HEC-1A/V or HEC-1A/OE sections were stained with EMP2 antisera or control antisera. Magnification, \(\times 20\). C and D, at day 31, mice were euthanized and tumor histology was assessed by H&E staining. Representative of excised tumors (left; bar, mm) and the corresponding histology (right; magnification, \(\times 40\)) for HEC-1A/V (C) and HEC-1A/OE (D). *, \(P < 0.05\), comparison by Student's \(t\) test.
other molecules (48–50). Thus, several lines of investigation might be pursued to determine mechanism by which anti-EMP2 diabodies elicit apoptosis.

In conclusion, treatment of endometrial adenocarcinoma cells with our highly specific anti-EMP2 diabody resulted in a significant increase in caspase-dependent apoptotic cell death in vitro and a reduction in tumor volume in vivo. These data, along with our previous finding that EMP2 positively correlates with the tumor phenotype of endometrial cancer, suggest that EMP2 may serve as a therapeutic target. Additional experiments will be necessary to determine the preclinical utility of EMP2 diabodies for endometrial cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Diabodies Targeting Epithelial Membrane Protein 2 Reduce Tumorigenicity of Human Endometrial Cancer Cell Lines

Kaori Shimazaki, Eric J. Lepin, Bo Wei, et al.


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