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Cancer Therapy: Preclinical

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EphA2 Targeted Chemotherapy Using an Antibody Drug Conjugate in Endometrial Carcinoma

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Abstract

Purpose: EphA2 overexpression is frequently observed in endometrial cancers and is predictive of poor clinical outcome. Here, we use an antibody drug conjugate (MEDI-547) composed of a fully human monoclonal antibody against both human and murine EphA2 (1C1) and the tubulin polymerization inhibitor monomethylauristatin F.

Experimental Design: EphA2 expression was examined in endometrial cancer cell lines by Western blot. Specificity of MEDI-547 was examined by antibody degradation and internalization assays. Viability and apoptosis were investigated in endometrial cancer cell lines and orthotopic tumor models.

Results: EphA2 was expressed in the Hec-1A and Ishikawa cells but was absent in the SPEC-2 cells. Antibody degradation and internalization assays showed that the antibody drug conjugate decreased EphA2 protein levels and was internalized in EphA2-positive cells (Hec-1A and Ishikawa). Moreover, *in vitro* cytotoxicity and apoptosis assays showed that the antibody drug conjugate decreased viability and increased apoptosis of Hec-1A and Ishikawa cells. *In vivo* therapy experiments in mouse orthotopic models with this antibody drug conjugate resulted in 86% to 88% growth inhibition ($P < 0.001$) in the orthotopic Hec-1A and Ishikawa models compared with controls. Moreover, the mice treated with this antibody drug conjugate had a lower incidence of distant metastasis compared with controls. The antitumor effects of the therapy were related to decreased proliferation and increased apoptosis of tumor and associated endothelial cells.

Conclusions: The preclinical data for endometrial cancer treatment using MEDI-547 show substantial antitumor activity. *Clin Cancer Res; 16(9); 2562–70. ©2010 AACR.*

Endometrial cancer is the most common gynecologic malignancy and the fourth most common cancer in North American and European women (1). Overall prognosis for these women is excellent as the majority of patients present with early-stage disease that is confined to the uterus at the time of hysterectomy, leading to 5-year survival rates of >70% (2). Unfortunately, women with recurrent or advanced-stage disease have a much poorer prognosis, with a median survival of ~12 months (3). The mainstay of treatment for these women remains systemic therapy in the form of hormonal agents or cytotoxic chemotherapy, and whereas some responses have been documented, the treatment programs are associated with intolerable side effects and infrequent durable remission (4). Therefore, newer and more effective targeted therapies are urgently needed.

Efforts to improve the cytotoxic action of monoclonal antibodies (mAb) and consequently their therapeutic effectiveness have focused on conjugates with highly toxic substances, including radioisotopes and cytotoxic agents (5). These conjugates can deliver a toxic load selectively to the tumor site while generally sparing normal tissues. To date, just two radioimmunoconjugates (ibritumomab tiuxetan and tositumomab) and one chemoimmunoconjugate (gemtuzumab ozogamicin) have been approved in the United States for limited use in refractory hematologic cancers (6–8). Thus, mAb conjugates can be a viable approach to killing tumor cells in hematologic malignancies.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

EphA2 overexpression is frequently observed in endometrial cancers and is predictive of poor clinical outcome. Due to the relatively low expression in normal adult tissues, EphA2 may represent a novel target for molecularly targeted delivery of cytotoxic agents. Here, we use an antibody drug conjugate (MEDI-547) composed of a fully human monoclonal antibody against both human and murine EphA2 (1C1) and the tubulin polymerization inhibitor monomethylauristatin F. MEDI-547 specifically bound to and was internalized by EphA2-positive cells (Hec-1A and Ishikawa). Moreover, *in vitro* cytotoxicity and apoptosis assays showed that MEDI-547 decreased viability and increased apoptosis of Hec-1A and Ishikawa cells. *In vivo* therapy experiments in mouse orthotopic models with MEDI-547 resulted in significant growth inhibition in the orthotopic Hec-1A and Ishikawa models.

The Eph receptors are the largest family of tyrosine kinases and are divided into two subclasses based on interaction with their ligands, ephrin-A and ephrin-B (9). There is growing evidence that several Eph receptors play critical roles in cancer development and progression (10). Moreover, high expression of EphA2 was detected in human endometrial carcinoma, which was significantly associated with poor clinical outcome (11). In normal adult tissues, EphA2 expression is either absent or present at low levels in only a few epithelial tissues (9); therefore, EphA2 could be an ideal therapeutic target in patients with advanced or recurrent endometrial cancer.

Recently, an EphA2 targeted antibody drug conjugate with a fully human mAb (1C1) has been developed, which selectively binds both the human and rodent EphA2 receptor. This antibody is conjugated to the microtubule inhibitor monomethylauristatin F (MMAF) using a stable maleimidocaproyl (mc) linker (MEDI-547; refs. 12–14). The purpose of our study was to determine the biological activity of this antibody drug conjugate in endometrial cancer using orthotopic endometrial cancer models.

Materials and Methods

Cell lines and cultures. Endometrial cell lines Ishikawa, Hec-1A, and KLE were maintained and propagated in MEM (Ishikawa), McCoy's 5A (Hec-1A), and 1:1 DMEM:F12 (KLE) media supplemented with 10% fetal bovine serum and 1% penicillin (Life Technologies) at 37°C (15). The human papillary serous endometrial carcinoma cell line SPEC-2 was maintained in a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and a 2-fold vitamin solution (Life Technologies Laboratories; ref. 16). The Ishikawa and Hec-1A cell lines were obtained from Dr. Russell Broaddus (M.D. Anderson Cancer Center), and SPEC-2 was from Dr. Isaiah J. Fidler (M.D. Anderson Cancer Center). The KLE cell line was obtained from the American Type Culture Collection. All experiments were conducted at 70% to 80% confluent cultures. Before *in vivo* injection, cells were trypsinized, centrifuged at 1,000 rpm for 7 minutes at 4°C, washed twice with HBSS, and resuspended in HBSS for intrauterine injections. Both cell lines were tested and found to be negative for murine antigen reactivity and Mycoplasma species before injection into mice.

Antibodies and antibody drug conjugates. 1C1 (fully human mAb recognizing both human and murine EphA2), control IgG-mcMMAF (nonbinding specific IgG mAb conjugated to MMAF via the mc linker), and MEDI-547 (1C1 conjugated to MMAF via the mc linker) were provided by MedImmune, LLC. The antibody description and the details of the conjugation reaction have been described previously (12).

Western blot. The preparation of cultured cell lysates has been described previously (17, 18). Briefly, protein concentrations were determined using a bicinchoninic acid protein assay reagent kit (Pierce Biotechnology), and aliquots of 20 μg protein were subjected to gel electrophoresis on 10% SDS-polyacrylamide gels. The proteins were then transferred to a nitrocellulose membrane (Millipore) and then incubated overnight at 4°C with primary antibody (mouse anti-human/mouse EphA2 mAb; clone D7, Upstate) after washing with TBST. The membranes were incubated with 1 μg/mL horseradish peroxidase–conjugated horse anti-mouse IgG (Amersham). Horseradish peroxidase was visualized by use of an enhanced chemiluminescence detection kit (Pierce; refs. 18, 19).

Antibody internalization. Procedures for antibody internalization after treatment with MEDI-547 in Hec-1A and Ishikawa cells were done as described previously (12). Briefly, viable cells (0.5×10^6) were aliquoted into wells of a 96-well plate in 100 μL of growth media. The cells were centrifuged at 1,500 rpm for 5 minutes and labeled with primary antibody drug conjugates by resuspension in 100 μL PBS containing 5 μg of MEDI-547 or control IgG-mcMMAF and incubated for 30 minutes at 4°C. Cells were then washed twice with PBS, and cell surface–bound primary antibody drug conjugates were allowed to internalize by resuspending the cells in 100 μL of growth media and incubation at 37°C/5% CO_2 for 30 minutes or on ice as negative control. Subsequent to internalization, cells were fixed (4% paraformaldehyde, 20 minutes at room temperature) and permeabilized (0.5% Triton X-100, 5 minutes at room temperature). Cells were then labeled with secondary AlexaFluor 488 goat anti-human IgG antibody (Biosource) by resuspension in 100 μL PBS + 2% fetal bovine serum containing 1 μg of secondary antibody and incubated for 30 minutes at 4°C (12).

Cytotoxicity assay. The cytotoxic effects of 1C1, control IgG-mcMMAF, and MEDI-547 were determined by the MTT uptake assay as described previously (20).

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Analysis of cell apoptosis. The relative percentage of apoptotic cells was assessed at three time points (24, 48, and 72 hours) using the Annexin V–FITC apoptosis detection kit-1 (BD Pharmingen) according to the manufacturer's protocol. Briefly, Hec-1A and Ishikawa cells were washed twice in PBS, and the pellet was resuspended in Annexin V binding buffer at a concentration of 10^6 cells/mL. Annexin V FITC and propidium iodide were added (5 μL to each per 10^5 cells). Samples were mixed gently and incubated for 15 min at room temperature in the dark before fluorescence-activated cell sorting analysis.

Animal care and orthotopic implantation of tumor cells. Female athymic mice (NCr-*nu*) were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center. The mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with the current regulations and standards of U.S. Department of Agriculture, U.S. Department of Health and Human Services, and NIH. All studies were approved and supervised by the University of Texas M.D. Anderson Cancer Center Institutional Animal Care and Use Committee. The mice used in these experiments were 8 to 12 weeks old. To produce tumors, Hec-1A and Ishikawa cells (both 4.0×10^6 per 50 μL HBSS; Life Technologies Invitrogen; ref. 15) or SPEC-2 cells (2.0×10^6 per 50 μL HBSS; ref. 16) were injected into the mice. Before injection, mice were anesthetized with isoflurane inhalation (Baxter) and a 0.5-cm incision was made in the right lower flank to optimize exposure to the right uterine horn. The distal portion of the horn was then identified and pulled to the incision for exposure. A single-cell suspension of 50 μL was then injected into the lumen of the uterine horn. The injection site was closely monitored during and following injection to ensure that no spillage occurred into the peritoneal cavity (15). The incision was then closed with staples. Mice (n = 10 per group) were monitored daily for adverse effects

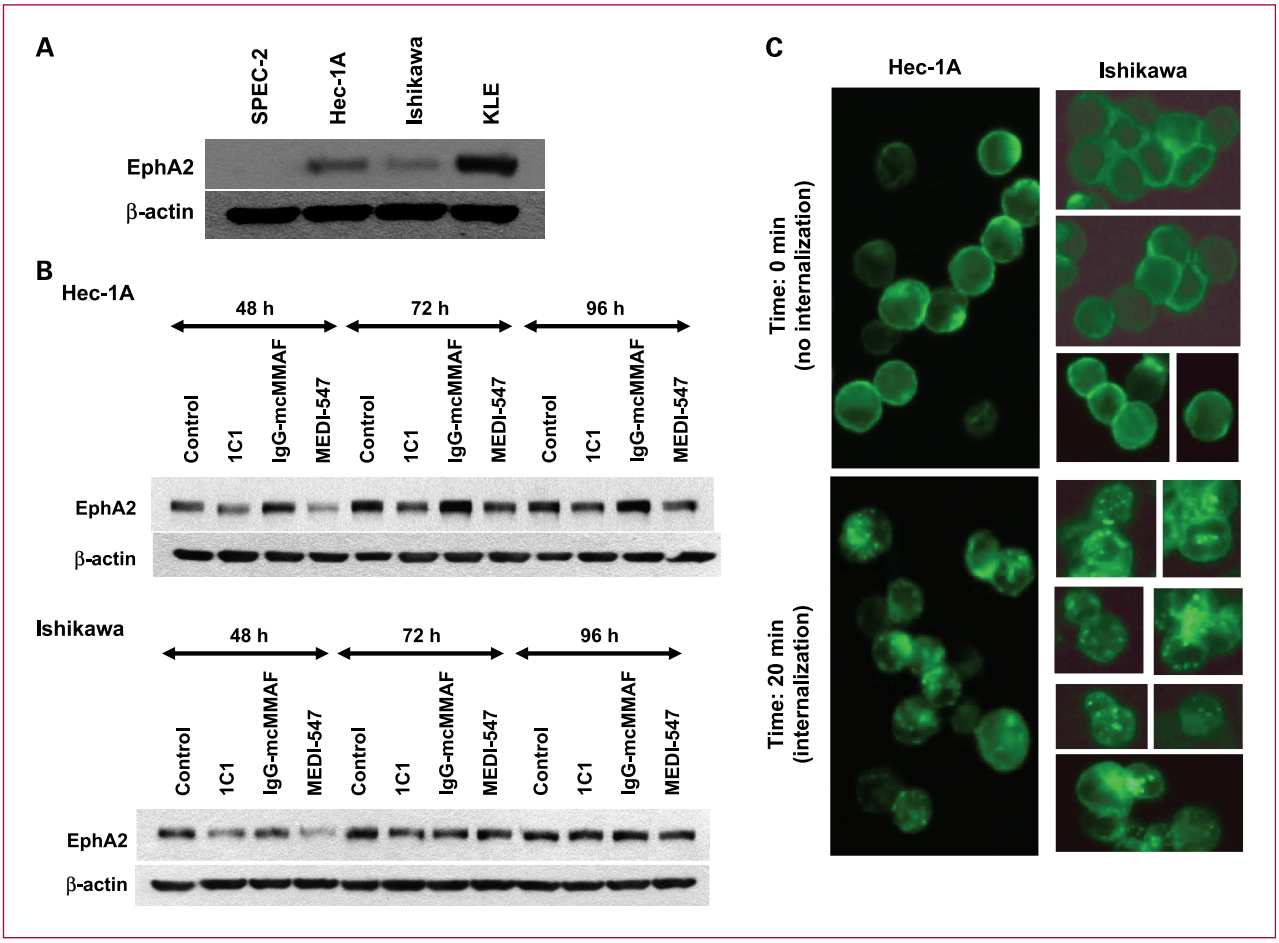

Fig. 1. EphA2 expression in endometrial cancer cell lines and the assessment of antibody degradation and internalization of EphA2 antibody drug conjugates. A, EphA2 expression in several endometrial cancer cells was assessed by Western blot. B, effect of 1C1 or MEDI-547 on EphA2 levels in Hec-1A and Ishikawa cells was assessed by Western blot. Cells were treated with PBS (control), 1C1, control IgG-mcMMAF, or MEDI-547 (each at 100 ng/mL) for 48, 72, or 96 hours. C, internalization of EphA2 on Hec-1A and Ishikawa cells treated with MEDI-547. Cells were immunostained for EphA2 with PBS containing MEDI-547 (5 μg) and then incubated for 20 minutes at 4°C. The experiment was done thrice.

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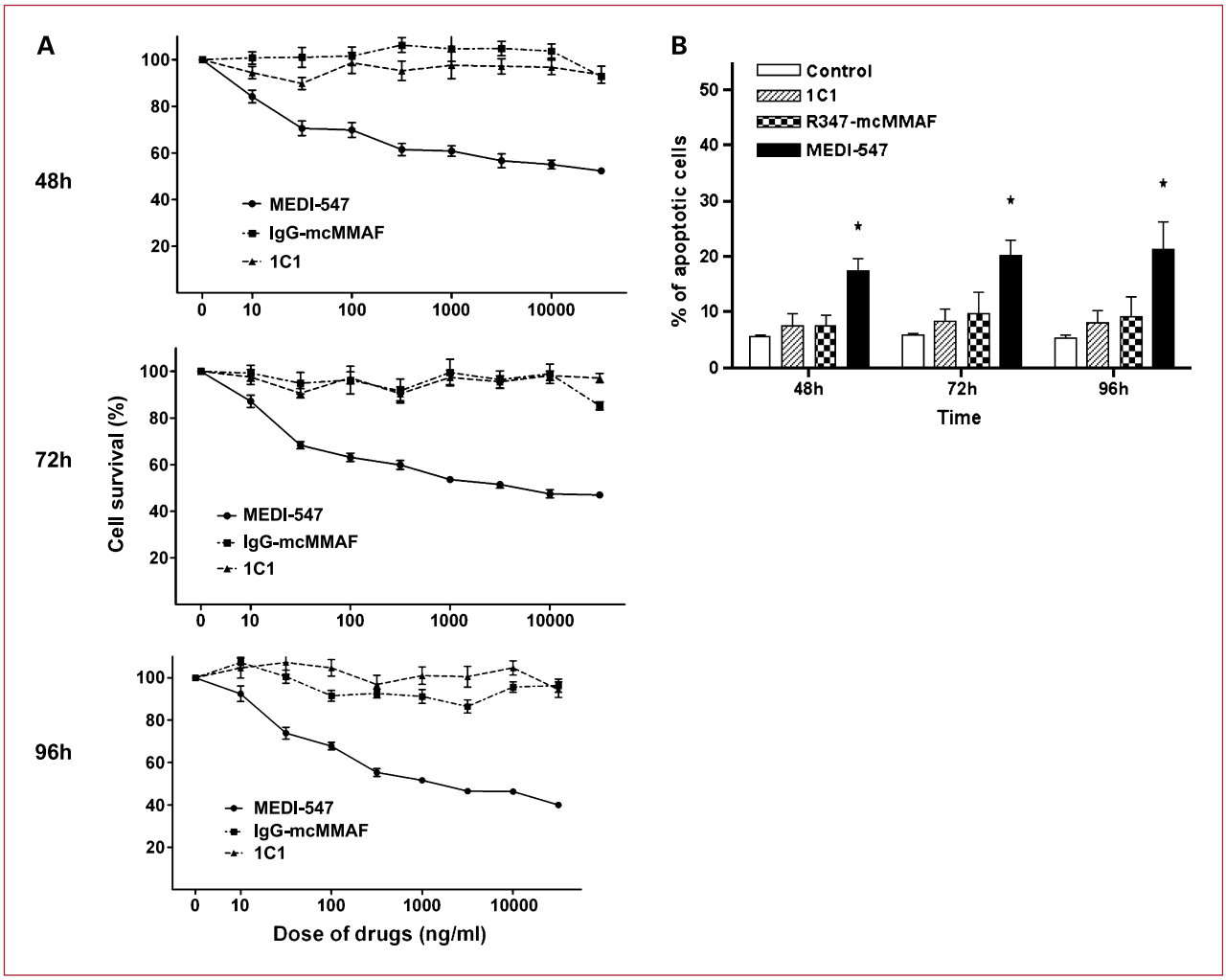

Fig. 2. Effect of MEDI-547 on *in vitro* tumor cell viability. A, viability of cultured Hec-1A cells after treatment with 1C1 or antibody drug conjugates (IgG-mcMMAF or MEDI-547). Viability was assessed with the MTT assay at 48, 72, or 96 hours after treatment with 1C1, control IgG-mcMMAF, or MEDI-547, respectively, at 10 to 50,000 ng/mL. Results were confirmed with duplicate experiments. Bars, SEM. B, apoptotic cells as detected by flow cytometry. Induction of apoptosis in Hec-1A cells after treatment with 500 ng/mL of PBS (control), 1C1, IgG-mcMMAF, or MEDI-547 for 24 to 72 hours. Apoptosis was examined by the Annexin V and propidium iodide dual staining assay, as detected by flow cytometry. The ratio of positive-staining cells was calculated and presented as mean ± SD. *, $P < 0.01$. Each experiment was preformed twice with similar results in both experiments.

of therapy and were sacrificed when any of the mice seemed moribund. Total body weight, tumor incidence and mass, and the number and location of tumor nodules were recorded. Tumors were fixed in formalin and embedded in paraffin or snap frozen in optimal cutting temperature media in liquid nitrogen.

Therapy for established endometrial tumors in nude mice. The antibody drug conjugate was dosed using 3 mg/kg i.p. injections weekly (14). Therapy experiments were designed using human endometrial cancer cell lines Hec-1A, Ishikawa, and SPEC-2. Following cell line injection, mice were randomized into four treatment groups: (*a*) control, 200 μL PBS (i.p., weekly); (*b*) 1C1, 3 mg/kg in 200 μL PBS (i.p., weekly); (*c*) control IgG-mcMMAF, 3 mg/kg in 200 μL PBS (i.p., weekly); and (*d*) MEDI-547, 3 mg/kg in 200 μL PBS (i.p., weekly). Therapy was initiated 2 weeks following cell line injection (15). Mice were monitored for adverse effects and sacrificed by cervical dislocation 6 to 7 weeks following initiation of treatment.

Immunohistochemistry. Procedures for immunohistochemical analysis of EphA2 and proliferating cell nuclear antigen (PCNA) were done as described previously (18, 21).

Immunofluorescence double staining for CD31 and terminal deoxynucleotidyl transferase-mediated nick end labeling. Frozen tissues were used for CD31/terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) immunofluorescence double staining as described previously (22). The quantification of apoptotic endothelial cells was calculated by the number of apoptotic endothelial cells in 10 random fields at ×200 magnification.

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Statistical methods. For animal experiments, 10 mice were assigned per treatment group. This sample size gave 80% power to detect a 50% reduction in tumor weight at a 5% level of statistical significance. Mouse and tumor weights and the number of tumor nodules for each group were compared using ANOVA and post-hoc analysis (Dunnett's method). Normality was tested by Kolmogrov-Smirnov test. Variables not meeting the criteria for normality were interrogated using the Mann-Whitney rank-sum test using the Statistical Package for the Social Sciences (SPSS, Inc.). A P value of <0.05 was considered statistically significant.

Results

EphA2 degradation and internalization of MEDI-547. Among the endometrial cancer cell lines tested, EphA2 protein expression was detected in the KLE, Hec-1A, and Ishikawa cells but was absent in the SPEC-2 cells (Fig. 1A). Because agonist antibodies can cause internalization and degradation of EphA2, we first asked whether MEDI-547 would affect EphA2 levels. EphA2 expression showed the greatest decrease following treatment with 1C1 or MEDI-547 compared with control (PBS) or control IgG-mcMMAF in Hec-1A and Ishikawa cells at 48 hours after treatment and gradual return of expression by 96 hours (Fig. 1B). After stimulation of the EphA2 receptor on Hec-1A and Ishikawa cells with MEDI-547 for 20 minutes, antibody internalization was detected by immunofluorescence (Fig. 1C). These results were not observed in the EphA2-negative SKMel 28 cells (data not shown).

In vitro ***sensitivity of endometrial cancer cells to MEDI-547.*** We next tested the effect of MEDI-547 on *in vitro* viability of the EphA2-positive Hec-1A and Ishikawa cells. The effect of MEDI-547 was tested at doses ranging

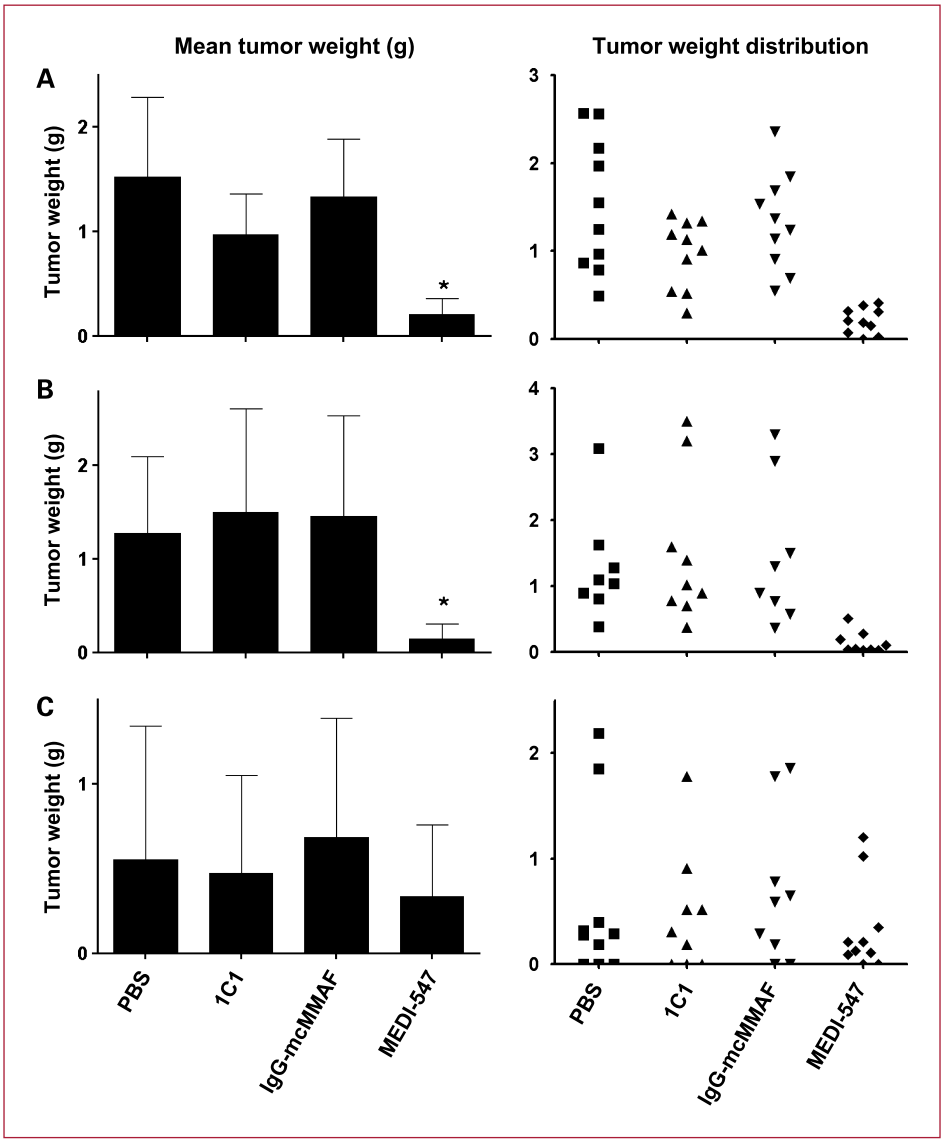

Fig. 3. Effect of MEDI-547 therapy on endometrial cancer growth. Mice inoculated with Hec-1A (A), Ishikawa (B), or SPEC-2 (C) cells received PBS (control), 1C1, control IgG-mcMMAF, or MEDI-547 (each at 3 mg/kg) after 2 weeks following cell line injection. Animals from all groups were sacrificed when control animals became moribund (5-6 weeks after initiating therapy depending on the cell line used). All tumors were harvested; mean tumor weight and its distribution were recorded. Columns, mean tumor weights for each samples group; bars, SD. *, $P < 0.01$.

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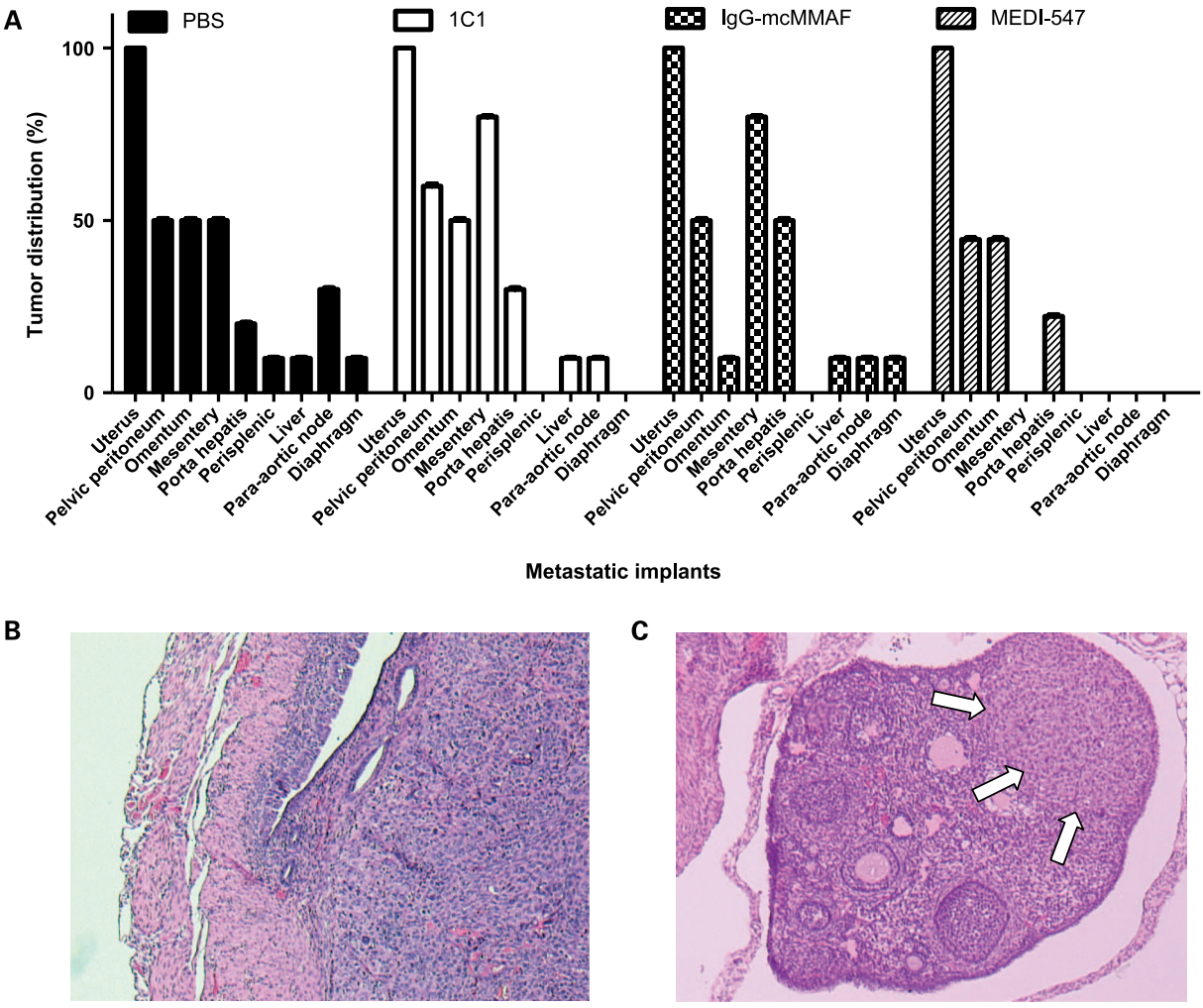

Fig. 4. Tumor distribution of Hec1A mouse model. A, MEDI-547 treated group had relatively less loci of distant metastasis, especially mesentery, liver, and para-aortic nodes. Histologic slides showed that the endometrial cancer had developed from the uterine cavity (B) and showed ovarian metastasis (arrowheads; C).

from 10 to 50,000 ng/mL. In the Hec-1A cells compared with controls (either 1C1 or control IgG-mcMMAF), growth was significantly inhibited by MEDI-547 in a dose-dependent manner (Fig. 2A). Similar results were noted with the Ishikawa cells (Fig. S1A). But there was no effect in EPhA2-negative SPEC-2 cells (Supplementary Fig. S2). Given the decrease in cell viability following treatment, we next asked whether the effects were apoptotic in nature. Treatment with MEDI-547 showed a significant increase in apoptosis compared with treatment with the controls in the Hec-1A ($P < 0.05$; Fig. 2B) and Ishikawa ($P < 0.05$, Fig. S1B) cells.

In vivo *tumor growth inhibition of MEDI-547–treated endometrial carcinoma.* On the basis of the observed effects for cytotoxicity and apoptosis on endometrial cancer cells, we next did several *in vivo* experiments using an orthotopic mouse model of uterine cancer to examine the potential therapeutic efficacy of MEDI-547. Mice injected with either Hec-1A or Ishikawa were assigned to one of four groups ($n = 10$ mice per group): (*a*) PBS; (*b*) 1C1, 3 mg/kg weekly; (*c*) control IgG-mcMMAF, 3 mg/kg weekly; or (*d*) MEDI-547, 3 mg/kg weekly. Following 5 to 6 weeks of therapy, the mice were sacrificed and necropsies were done. Prolonged MEDI-547 therapy led to a significant reduction in tumor growth in the Hec-1A and Ishikawa models compared with control (PBS), 1C1, or control IgG-mcMMAF (each $P < 0.01$; Fig. 3A and B, respectively). We next asked whether the MEDI-547 has antiangiogenic effects *in vivo* because it also recognizes murine EphA2 (18). To address this question, we used the EphA2-negative SPEC-2 cells (Fig. 1A). The tumor vasculature is known to express higher EphA2 levels compared with normal endothelial

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cells (18). Although the mean tumor weight of MEDI-547–treated group was lower than the other groups, the difference did not reach statistical significance (Fig. 3C). No obvious signs of toxicity were observed in the treatment groups (i.e., body weight loss).

In the Hec-1A model, metastases developed to the pelvis, omentum, mesentery, porta hepatis, perisplenic area, liver, para-aortic lymph nodes, and diaphragm. We also examined whether MEDI-547 therapy could reduce metastatic spread. When we assessed the number of metastases by site (n = 10 per group), treatment with MEDI-547 resulted in a substantially lower frequency of metastases compared with the 1C1 or control IgG-mcMMAF controls, especially in mesentery, liver, and para-aortic nodes (Fig. 4A). And in a representative sample, histologic slides showed that the endometrial cancer had developed from the uterine cavity (Fig. 4B) and showed ovarian metastasis (Fig. 4C).

Effect of MEDI-547 on cell proliferation, apoptosis, and antiangiogenesis. To determine potential mechanisms underlying the antitumor activity of MEDI-547 treatment, we examined the effect of MEDI-547 therapy on tumor cell proliferation by using PCNA staining. In the Hec-1A model, tumor cell proliferation was significantly reduced following MEDI-547 treatment compared with controls ($P < 0.05$; Fig. 5). Similar results were noted in the Ishikawa model ($P < 0.05$; Supplementary Fig. S3). TUNEL staining revealed that MEDI-547 treatment also resulted in significantly increased apoptosis in the Hec-1A ($P < 0.05$; Fig. 5) and Ishikawa ($P < 0.05$; Supplementary Fig. S3) models. To test for potential direct effects on the tumor vasculature, we did dual immunofluorescence (CD31/TUNEL) staining following short-term treatment. There was a significant increase in endothelial cell apoptosis in the MEDI-547 group in both Hec-1A ($P < 0.05$; Fig. 5) and Ishikawa ($P < 0.05$; Supplementary Fig. S3) models. These data suggest that the MEDI-547 therapy exerts antitumor effects by both direct and indirect effects on tumor growth. Moreover, in the EphA2-negative SPEC-2 model, whereas tumor cell PCNA and TUNEL expression was not significantly lower, there was significantly greater endothelial cell apoptosis with MEDI-547 treatment ($P < 0.05$; data not shown).

Discussion

The identification of effective therapies for the treatment of endometrial cancer is critical. Although current therapies have included various chemotherapeutics, recent radiation and hormonal therapy reports suggest that

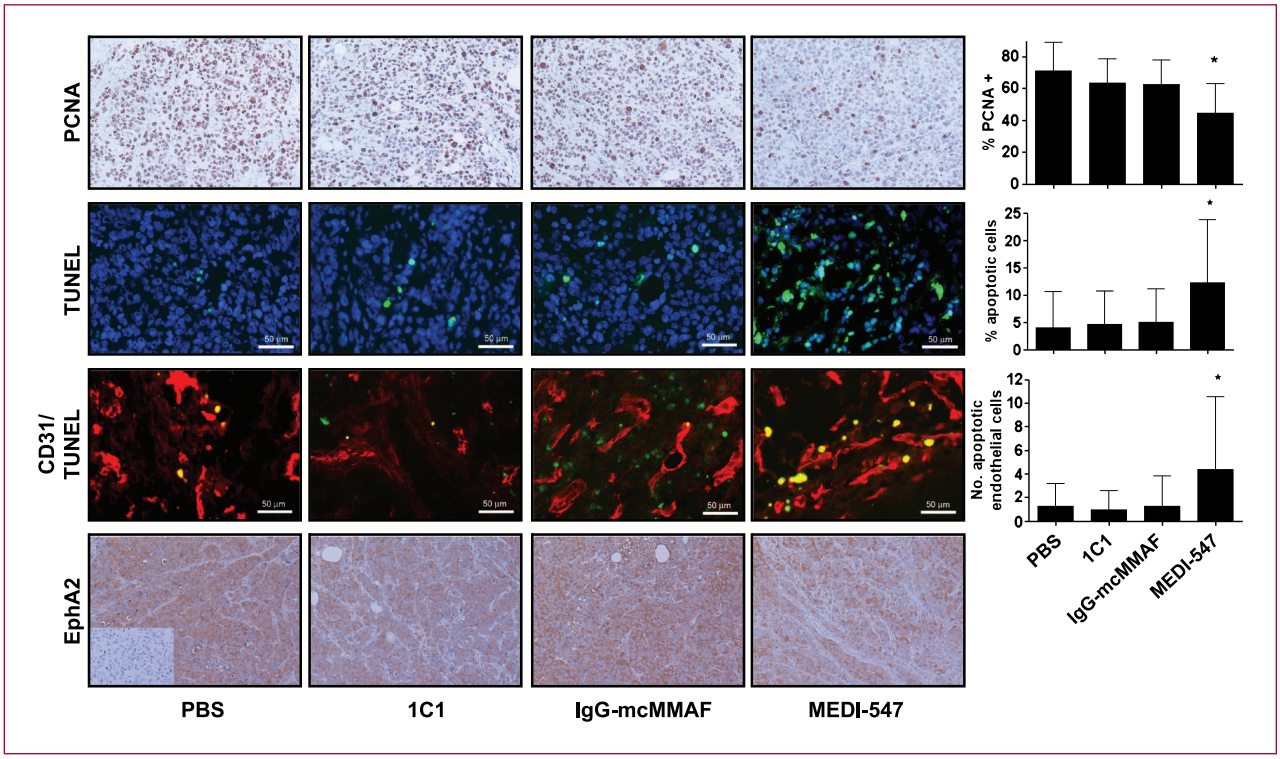

Fig. 5. *In vivo* effects of MEDI-547 on proliferation and apoptosis of tumor or endothelial cells in the Hec-1A model. A, tumor sections were stained with PCNA to reveal tumor cell proliferation. PCNA-positive cells were counted and graphed. *, $P < 0.05$. B, tumor sections were stained with Hoechst (blue) and TUNEL (green) using double immunofluorescence staining. The tumor cells undergoing apoptosis show green fluorescence. C, tumor sections were stained with CD31 (red) and TUNEL (green) using double immunofluorescence staining. Colocalization of endothelial cells undergoing apoptosis shows yellow fluorescence. D, EphA2 expression in the tumor tissues taken at the time of sacrifice (3 days after injection of last dose) was assessed by immunohistochemistry. Inset in PBS panel represents a negative control. Error bars, SD. *, $P < 0.05$.

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biologics, such as bevacizumab or cetuximab, may be efficacious in endometrial cancer (23, 24). The EphA2 antibody drug conjugate (MEDI-547) selectively delivers a tubulin polymerization inhibitor to EphA2-expressing tumors resulting in cell death and the inhibition of tumor growth.

The key findings from this study are that MEDI-547 effectively inhibits uterine cancer growth in orthotopic xenograft tumor models without overt signs of toxicity (i.e., weight loss). Our data indicate that MEDI-547 is internalized by the EphA2-positive cells, resulting in cytotoxicity. Moreover, MEDI-547 shows therapeutic activity in orthotopic animal models by both direct and possibly indirect effects on tumor cells.

The microtubule inhibitors paclitaxel and docetaxel, in combination therapy, are used to treat endometrial cancer patients and have shown some promise. We would anticipate that MEDI-547 treatment may be useful for endometrial cancer patients by delivery of the microtubule inhibitor (MMAF) to the tumor. Derivatives of MMAF have been successfully used as potent cytotoxic agents that are delivered by conjugated antibodies (25). Various linker and auristatin combinations can specifically kill tumor cells after internalization through cell surface antigens, such as CD30 (26), CD70 (27), and CD79 (28). The covalent linkage is highly stable until the antibody-drug conjugate enters lysosomal vesicles, which allows for drug release in the cytoplasm and the disruption of microtubule polymerization (29–31). In addition to endometrial cancer, MEDI-547 was previously reported to effectively inhibit the growth of human prostate cancer xenografts (12).

We have recently reported the use of a novel orthotopic model for uterine cancer that recapitulates the pattern of spread seen in endometrial cancer patients (15). A major drawback of using s.c. xenograft models is that the microenvironment is quite different compared with tumors grown in the native organ (32, 33). In the current study, we used the orthotopic uterine cancer model using Hec-1A or Ishikawa cells. Following tumor growth within the uterus, there was spread to multiple locations in the abdominal cavity. MEDI-547 treatment was effective in reducing metastatic spread as well as primary tumor and total tumor weight (Fig. 4).

EphA2 protein has been reported to be overexpressed in ~50% of ovarian, prostate, colon, and brain cancers (34). Whereas the expression of EphA2 receptor in human cancer has been well established, little is known about the normal adult tissue expression and function (12). Imaging studies using a radiolabeled version of the highly selective anti-EphA2 antibody 1C1, revealed preferential distribution to tumors with no accumulation in any normal mouse or rat normal tissues (35), suggesting that targeting of the MEDI-547 to normal mouse tissues is minimal.

Disclosure of Potential Conflicts of Interest

D.J., J.G., and S.M. are all employees of MedImmune LLC. The other authors disclosed no potential conflicts of interest.

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