Published in final edited form as:

Cancer. 2015 February 1; 121(3): 403–412. doi:10.1002/cncr.29062.

Solitomab, an EpCAM/CD3 bispecific antibody (BiTE®), is highly active against primary chemotherapy resistant ovarian cancer cell lines *in vitro* and fresh tumor cells *ex vivo*

Diana P. English, MD^{1,*}, Stefania Bellone, PhD^{1,*}, Carlton L. Schwab, MD¹, Dana M. Roque, MD¹, Salvatore Lopez, MD¹, Ileana Bortolomai, PhD¹, Emiliano Cocco, PhD¹, Elena Bonazzoli, MS¹, Sudeshna Chatterjee, MD¹, Elena Ratner, MD¹, Dan-Arin Silasi, MD¹, Masoud Azodi, MD¹, Peter E. Schwartz, MD¹, Thomas J. Rutherford, MD¹, and Alessandro D. Santin, MD¹

¹Department of Obstetrics, Gynecology & Reproductive Sciences, Yale University School of Medicine, New Haven, CT

Abstract

Background—Solitomab is a novel bispecific single-chain antibody which targets EpCAM on tumor cells and also contains a CD3 binding region. We evaluated *in vitro* activity of solitomab against primary chemo-resistant epithelial ovarian carcinoma cell lines as well as malignant cells in ascites.

Methods—EpCAM expression was evaluated by flow cytometry in 5 primary ovarian cancer cell lines and 42 fresh ovarian tumor cell cultures from ascites of patients with mainly advanced or recurrent chemo-resistant disease. The potential activity of solitomab against EpCAM + tumor cells was evaluated by flow-cytometry, proliferation and 4-hr chromium-release cell-mediated cytotoxicity assays.

Results—Expression of EpCAM was detected by flow cytometry in approximately 80% of fresh ovarian tumors and primary ovarian tumor cell lines tested. EpCAM + chemo-resistant cell lines were found resistant to NK or T-cell-mediated killing after exposure to peripheral blood lymphocytes (PBL) in 4-hour chromium-release assays (mean killing $\pm$ SEM, $3.6 \pm 0.7\%$ after incubation of EpCAM + cell lines with control BiTE). In contrast, after incubation with solitomab, EpCAM + chemo-resistant cells became highly sensitive to T cell cytotoxicity (mean killing $\pm$ SEM of $28.2 \pm 2.05\%$; $P < 0.0001$) by PBL. *Ex vivo* incubation of autologous tumor associated lymphocytes (TAL) with EpCAM expressing malignant cells in ascites with solitomab, resulted in a significant increase in T-cell activation markers, and a reduction in number of viable ovarian tumor cells in ascites ($P < 0.001$).

Corresponding Author: Alessandro D. Santin, MD, 333 Cedar Street, LSOG 305, PO Box 208063, New Haven, CT 06520-8063, USA, Telephone: 203-737-4450, Fax: 203-737-4339, alessandro.santin@yale.edu.

*Dr. English and Dr. Bellone equally contributed to this manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest or previous publication and fulfill all conditions required for authorship.

The authors declare no conflict of interest or previous publication and fulfill all conditions required for authorship. No financial disclosures for any of the authors other than funding source stated above.

Conclusions—Solitomab may represent a novel, potentially effective agent for treatment of chemo-resistant ovarian cancer overexpressing EpCAM.

Keywords

EpCAM; CD3; T-lymphocyte; Bispecific antibody; Ovarian cancer

INTRODUCTION

Epithelial ovarian carcinoma (EOC) remains the gynecologic malignancy with the highest mortality rate ¹. Two-thirds of patients have advanced disease at the time of diagnosis and, unfortunately, the majority of patients will recur after an initial response to the combination of maximal cytoreductive surgery and combined platinum-paclitaxel-based chemotherapy². The identification of novel therapies against chemotherapy resistant/recurrent ovarian cancer remains a high priority.

With the use of high-throughput genomic analysis, our group and others have recently evaluated the genetic fingerprints of primary and metastatic ovarian carcinoma with the hope of identifying novel molecular diagnostic and therapeutic markers ^{3–5}. These studies consistently demonstrated epithelial cell adhesion molecule (EpCAM, also known as TROP-1 or TACSTD1), a calcium-independent hemophilic cell adhesion molecule of 39 to 42 kd, as one of the top differentially expressed genes in all epithelial ovarian cancer types tested including serous, clear-cell, endometrioid and poorly differentiated tumors ^{3,4}.

Briefly, the EpCAM gene is located on chromosome 4q and contains 9 exons ⁶. It encodes for a protein consisting of an extracellular domain with 2 epidermal growth factor-like repeats, a transmembrane, and a short cytoplasmic domain of 26 amino acids. EpCAM promotes cell adhesion and the formation of EpCAM-mediated adhesions has negative regulatory effect on adhesions conferred by cadherins ^{6,7}. EpCAM is expressed at low levels on the basolateral and intercellular surface of simple, pseudo-stratified, and transitional epithelia, including most epithelial tissues in the female genital tract ⁸. The differentiation of tumor cells from normal cells is facilitated by assessment of homogeneity of EpCAM on the tumor cell, its glycosylation, as well as the level of EpCAM expression present ⁹. EpCAM is known to play an important role in multiple cell functions including cell signaling, cell migration, proliferation, and differentiation. Recent evidence has linked increased EpCAM expression with ovarian cancer progression ¹⁰. Importantly, because of its high expression on the cell surface of carcinomas, EpCAM represents an attractive target for immunotherapy ⁶.

In the last few years several EpCAM (CD326)/CD3-bispecific antibodies have demonstrated in multiple murine models the capability to eliminate established tumors, to redirect tumor associated T cells for lysis of metastatic human ovarian cancer tissue ^{11,12} and to effectively treat a variety of metastatic cancers ^{13–15}. Solitomab (MT110, AMG 110) is an EpCAM/CD3-bispecific single-chain antibody in development for the treatment of patients with multiple solid tumors expressing EpCAM (CD326) ¹¹. Solitomab has shown remarkable antitumor activity in preclinical ovarian tumor xenograft models due to the ability to engage

resting, polyclonal CD8+ and CD4+ T cells for highly potent redirected lysis of target tumor cells.

In this study we evaluated the *in vitro* activity of solitomab against multiple primary chemotherapy resistant epithelial ovarian carcinoma (EOC) cell lines as well as unmanipulated malignant tumor cells in ascitic fluid of patients harboring mainly advanced stage or chemotherapy resistant ovarian disease. Our results demonstrate impressive solitomab anti-tumor activity against chemotherapy-resistant EOC cell lines and tumor cells from the ascitic fluid of ovarian cancer patients.

METHODS

Patients and Sample Processing

All patients signed an informed consent form according to institutional guidelines and approval for this study was obtained from the institutional review board. Ascitic fluid samples were collected from 42 consecutive patients with cytologically confirmed ovarian cancer at the time of cytoreductive surgery or at the time of a diagnostic or therapeutic paracentesis. The ascitic fluid of all patients was tested for presence of EpCAM-positive ovarian cancer cells by flow cytometry. Random samples of ascites were also plated in 6-well microtiter plates for treatment using solitomab and a control non-specific antibody without prior processing. Cell number and viability were determined by flow cytometry as described below. Also a total of five primary ovarian cancer cell lines were established after sterile processing of surgical biopsy samples as described previously¹⁶. All primary ovarian cancer cell lines used in this study were found highly resistant to multiple chemotherapy drugs *in vitro* including carboplatin, cisplatin, paclitaxel, doxorubicin, ifofosfamide, gemcitabine and topotecan^{16,17}. Patient characteristics of all ovarian cancer cell lines and ascitic fluid effusates are described in Table 1 and 2.

Ex vivo therapy of malignant ascitic fluid samples

Malignant ascites from ovarian cancer patients were analyzed after *ex vivo* treatment with solitomab or a control bispecific antibody. The malignant ascites were plated in duplicate in 6-well flat microtiter plate. The ascites were treated with the bispecific antibody, solitomab (Amgen Research Munich GmbH, Munich, Germany) at a concentration of 1µg/ml for 5 days. As a control condition, these ascites were treated with control Bite huMEC14 also at a concentration of 1µg/ml. The effect of solitomab on the malignant ascites tumor cells was assessed by observation of induction of morphologic changes and extent of cytotoxicity, as well as, for evidence of T cell activation and induction of cytokine release as described below.

Flow cytometry

Characterization of EpCAM expression in malignant ascitic cells before treatment was performed by FACS analysis. The anti-human EpCAM-PE antibody clone 1B7 (eBioscience) was used for flow cytometry studies. The IgG1-PE antibody (BD Biosciences) was used as antibody isotype control for the anti-EpCAM antibody. The detection of the immune cell fractions was determined by using anti-CD8-PE and anti-CD4-PE antibodies.

Activation of immune cells was detected by anti-CD25 and anti-HLA-DR antibody. Analysis was conducted with FACScalibur flow cytometer with Cell Quest software (Becton Dickinson, Franklin lakes, NJ).

T cell stimulation assay

Solitomab induced T cell activation was measured by detecting CD25 protein surface expression and HLA-DR expression on CD8⁺ and CD4⁺ T cells by FACS. Solitomab mediated stimulation of T cells was calculated according to the following formula: Percentage of CD8⁺/CD25⁺ expression = [number of CD8⁺/CD25⁺ cells/ total number of CD8⁺ cells] × 100. Similarly, using the same equation the number of CD8⁺/HLA-DR⁺, CD4⁺/CD25⁺ and CD4⁺/ HLA-DR⁺ expression was calculated.

Cytokine analysis

The level of solitomab dependent cytokine induction was compared to the corresponding value of percentage of cytokine release in the control non-specific antibody control wells. This was performed by treating the solitomab and control non-specific antibody wells with phorbol myristate acetate and ionomycin followed by a 3 hour incubation period to allow for lymphocyte stimulation. Brefeldin A was added and a further incubation for 3 hours occurred in order to enhance intracellular cytokine staining signals. Cytokine analysis of the supernatants was performed by FACS analysis after adding anti-CD8-FITC antibody for surface staining followed by fixation, permeabilization and intracellular staining with anti-IL-4-PE antibody and anti-IFN gamma-PE antibody. Solitomab mediated release of each of these cytokines was calculated according to the following exemplary formula: Percentage of CD8⁺/ IFN gamma containing cells = [number of CD8⁺/ IFN gamma cells/ total number of CD8⁺ cells] × 100. Similar calculations were performed for CD4⁺ T cells (i.e., gated CD3⁺/CD8-T cells).

Proliferation assay of tumor associated T-lymphocytes (TAL) after the addition of EpCAM BiTE by CFSE Cell proliferation

Briefly, ascitic cells were harvested and washed twice with PBS and immediately stained with carboxyfluorescein succinimidyl ester (CFSE) (CellTrace CFSE Cell Proliferation Kit, Invitrogen, Carlsbad, CA) at a working concentration of 10 micromolar. The CFSE labeled cells were plated and cultured in the presence of Control Bite huMEC14 or solitomab for 5 days. Cells were collected and labeled with CD8 and CD4 (Becton Dickinson) and analyzed on a flow cytometer (FacsCalibur, Becton Dickinson) using Sciquest. The percentage and numbers of CD8⁺ and CD4⁺ T cells in the control BITE wells vs solitomab treated wells were calculated after analysis by flow cytometry to determine TAL proliferation induced in this co-culture system.

Tests for T cell mediated cytotoxicity

The standard 4-hour chromium (⁵¹Cr) release assay was used to measure the cytotoxic reactivity of Ficoll-Hypaque-separated peripheral blood lymphocytes (PBLs) from several healthy donors against all five OSC cell lines at effector to target ratios (E:T) of 10:1 and 20:1. The release of ⁵¹Cr from target cells was measured as evidence of tumor cell lysis after

exposure of the tumor cells to a concentration of 1 µg/ml of solitomab. The negative control conditions were the incubation of target cells alone or with PBL without BiTE antibody. As a positive control condition, 1% sodium dodecyl sulfate (SDS) was used to achieve complete lysis of target cells. The control BiTE huMEC14 at 1 µg/ml was used as the negative control for solitomab in this bioassay. The control BiTE antibody shared the CD3 binding arm with solitomab, but otherwise recognizes an herbicide as an irrelevant antigen instead of recognizing EpCAM. The percentage cytotoxicity of solitomab was calculated by the following formula: $\% \text{ cytotoxicity} = 100 \times (ES)/(T-S)$, where *E* is the experimental release, *S* is the spontaneous release by effector cells, *T* is the maximum release by target cells lysed with 1% SDS.

STATISTICS

Differences in EpCAM expression by flow cytometry were analyzed by the unpaired *t* test. The unpaired *t* test was used to evaluate the differences in ADCC levels in primary tumor cell lines treated with solitomab versus control BiTE huMEC14. T cell activation marker increase as well as cytokine release was analyzed by the paired *t* test. Statistical analysis was performed using SPSS version 18. A P-value of < 0.05 was considered as the level of statistical significance.

RESULTS

Epithelial cell adhesion molecule levels in ovarian carcinoma primary tumors and ascitic samples

We used flow cytometry to obtain highly sensitive measurements of EpCAM surface expression in five primary ovarian cell lines (4 established from patients harboring chemotherapy resistant disease)^{16,17} and in 42 ascitic fluid samples collected from advanced stage ovarian carcinoma patients. Table 1 and 2 depict the characteristics of the patients from which the samples were collected and shows the percentage of EpCAM positive cells and corresponding mean fluorescence intensity (MFI) in primary ovarian carcinoma cell lines and 42 ascitic fluid samples by flow cytometry. Amongst the primary cell lines, the percentage of EpCAM positive cells and EpCAM expression levels were found to be high in OSC ARK-1, OSC ARK-2, CC ARK-2, and A(PL)10 (Table 1). In contrast, low to negligible EpCAM expression was found in OSC ARK-3. Amongst the freshly collected ascitic fluid samples we found 85.7% (36 out of 42) of the samples harbor EpCAM positive tumor cells (cut-off for EpCAM tumor positivity arbitrary placed at 1 % EpCAM-positive tumor cells in the ascitic population). The percentage positive EpCAM cells in ascitic samples vary from 1% to 92.9% while the minimum to maximum MFI for EpCAM expression ranged from 95.6 to 4539.3 (Table 2). As noted in Table 2, there were ascitic samples with very low percentage of EpCAM positive tumor cells but with very high MFI. EpCAM expression of greater than 1% in ascitic samples was sufficient to note a response with solitomab (Table 2 and Figure 3).

Ovarian carcinoma cell lines are resistant to natural killer (NK) cell activity but sensitive to solitomab-mediated T cell cytotoxicity

Firstly, standard 4-hour chromium (^{51}Cr) release assay cytotoxicity titration was performed using solitomab (EpCAM BiTE®) from doses of 0 to 2.5 $\mu\text{g/ml}$ in several experiments aimed at determining the optimum dose of solitomab. From these experiments the 1 $\mu\text{g/ml}$ concentration emerged as the optimum concentration for tumor killing (Figure 1). All five primary ovarian cancer cell lines were evaluated for their sensitivity to NK and T-cells. These cell lines were exposed to peripheral blood lymphocytes collected from multiple healthy donors in several cytotoxicity assays. These peripheral blood lymphocytes typically contain between 20–25% CD8^+ T cells and 10–20% NK cells ($\text{CD56}^+/\text{CD16}^+$).

Ovarian cancer cell lines were found to be highly resistant to NK and T-cell-mediated killing with exposure to PBL at effector: target ratios of 1:10 and 1:20 (mean killing $\pm$ SEM, $3.6 \pm 0.7\%$ with range of killing 0–12.7% after incubation of EpCAM-positive cell lines with control BiTE). In contrast, significant killing was seen against EpCAM-positive cell lines after incubation with solitomab to mediate T cell cytotoxicity with a range of killing, 10–50.77%; mean killing $\pm$ SEM, $28.2 \pm 2.05\%$; $P < 0.0001$. As expected, the low/negative EpCAM expressing cell line (OSC ARK-3) demonstrated negligible levels of cytotoxicity with solitomab compared with other ovarian serous carcinoma cell lines (Figure 2).

Co-culture of solitomab (EpCAM BiTE) and tumor-associated lymphocytes triggers T-lymphocyte proliferation and cytotoxicity in a potentially immunosuppressive environment

T-lymphocyte proliferation and cytotoxicity was evaluated after co-culturing for 5 days with EpCAM-positive tumor cells with solitomab and autologous TAL in freshly collected ascitic fluid samples. We used CFSE and anti-CD4 and anti-CD8 antibodies to label lymphocytes and anti-EpCAM antibodies to label tumor cells to be counted by flow cytometry. At baseline we found a larger percentage of CD4^+ T cells in the ascites when compared to CD8^+ T cells (Mean percentage $\pm$ SD = CD4, 39.1 ± 15.4 vs CD8, 23.8 ± 11.8 , ratio 1.6). Incubation with solitomab when compared to control BiTE significantly increased CD8^+ T cell numbers in ascitic fluid cultures (15.4 ± 13.1 vs 33.1 ± 23.7 control BiTE vs solitomab, respectively, $P = 0.03$). The activated T-lymphocytes primed for tumor cell destruction by solitomab (see results below) also had a change in their morphologic appearance, as T-lymphocytes in the solitomab co-culture appeared larger than in the control BiTE (data not shown). Incubation with solitomab increased CD4^+ T cell number in most of the ascitic cultures when compared to control BiTE (26.9 ± 26.3 vs 42.2 ± 26.1 control BiTE vs solitomab, respectively), approaching statistical significance ($P = 0.06$).

The environment of ascitic fluid is one of a potentially immune-tolerance as depressed immune responses are commonly seen in advanced cases of cancer. Serum and ascites from patients with various types of cancer has been shown to contain factors which cause a non-specific suppression of various T- and B-lymphocyte responses and of natural killer (NK) cell activity. In light of this, the ability of solitomab to stimulate lymphocyte proliferation and cytotoxic activity in the immuno-tolerant ascitic fluid environment cannot be understated. Incubation for 5 days with solitomab significantly decreased the number of EpCAM-positive tumor cells in the freshly collected ascitic fluid samples derived from

patients harboring advanced/recurrent disease (decrease of EpCAM-positive tumor cells vs control BiTE = mean $35.7\% \pm 27.1$, range from 2.6–74.4%, $p < 0.0001$), Figure 3. Figure 4 shows a representative photograph of control BiTE treated ascites vs solitomab treated ascites under the microscope. A dramatic reduction in the number of viable tumor cells in the plate was detected in these experiments. In contrast, EpCAM negative ascitic tumors demonstrated negligible levels of T-lymphocyte cytotoxicity in the presence of solitomab (data not shown).

Activation of T-lymphocytes by *solitomab* (EpCAM BiTE®) in co-cultures with ovarian carcinoma cells

Next, we explored whether solitomab is capable of inducing T cell activation in the presence of ovarian tumor cells in ascitic fluid samples. CD8⁺ T and CD4⁺ T cells at baseline and after 5-day co-cultures with ascitic fluid and solitomab were analyzed by flow cytometry for the expression of the T-cell activation markers CD25 and HLA-DR. As demonstrated in Figure 5, we found a significant increase in both CD25 and HLA-DR protein surface expression on CD8⁺ and CD4⁺ T cells (Figure 5) in the solitomab treated wells when compared to the wells treated with the control BiTE® ($p < 0.001$). In contrast, EpCAM negative ascitic samples failed to demonstrate significant increase in both CD25 and HLA-DR protein surface expression on CD8⁺ T cells in the solitomab treated wells when compared to the wells treated with the control BiTE® ($p = 0.57$).

Cytokine release accompanying T lymphocyte activation

Next we investigated the level of the solitomab dependent cytokine induction compared to the cytokine release in the control non-specific BiTE® control wells. The flow analysis was performed after 5 days of co-incubation of solitomab with tumor cells and PBL before lymphocyte stimulation in the presence of brefeldin A as described in the Methods section. The analysis by FACS revealed that there was a significant increase in IFN-gamma production in EpCAM + ascites exposed to solitomab compared to control BiTE® wells in both CD8⁺ T and CD4⁺ T cells, $P = 0.04$ (Figure 6). In contrast, we were unable to demonstrate a significant increase in IFN-gamma production in EpCAM negative ascitic wells, $P = 0.41$. Also there was no significant increase in IL-4 cytokine release in solitomab exposed EpCAM positive or negative ascitic wells when compared to wells stimulated with control BiTE® ($p = 0.31$, data not shown).

DISCUSSION

Epithelial ovarian cancer is the leading cause of death from gynecologic cancers in the United States. Over 65% of women will present with advanced stage disease and many of these patients will eventually develop recurrent disease and succumb to chemotherapy resistant tumors. The development of novel therapies effective in the treatment of recurrent chemotherapy-resistant ovarian cancer remains an unmet medical need.

BiTE® antibodies are single-chain bispecific antibody constructs with specificity for CD3 on T cells and a surface antigen on tumor cells. By bridging cytotoxic T cells and cancer cells, BiTE® antibodies are capable of mounting a T cell response that is not restricted by T

cell receptor specificity, presence of MHC class I, generation and presentation of peptide antigen, or the need for T cell co-stimulation. In this study we explored the potential of solitomab, an EpCAM/CD3 bispecific antibody (BiTE®), as a novel therapeutic strategy against chemotherapy-resistant ovarian cancer cells.

We initially evaluated EpCAM expression in multiple primary ovarian cancer cell lines obtained from patients with chemotherapy resistant disease as well as in free ovarian cancer cells and spheroids formed *in vivo* in the ascitic fluid from ovarian cancer patients. Consistent with our previous results^{16,17}, we found EpCAM to be overexpressed in the majority of cell lines and ascitic fluid samples.

Importantly, we found primary ovarian cancer cell lines overexpressing EpCAM to be highly susceptible to ADCC mediated by solitomab. The cytotoxic activity of solitomab against a human colon cancer cell line and metastatic ovarian cancer cells in the presence of PBL has previously been demonstrated by Schlereth et al.¹². In our study, we extend the results of Schlereth et al. by evaluating the cytotoxic potential of solitomab against multiple biologically aggressive and highly chemotherapy-resistant ovarian cancer cell lines with serous and clear cell histology. We found all primary ovarian tumors tested showing high EpCAM expression to be highly susceptible to solitomab-mediated T cell killing in the presence of effector cells while negligible cytotoxicity was detected in the cell line with low level of EpCAM surface expression cells. It is worth noting that although all EpCAM-positive cell lines were resistant to natural killer cytotoxic activity (i.e., average killing at 20:1 effectors to target cell ratio in controls = 3.6%), solitomab-mediated T cell cytotoxicity resulted in killing of up to 50% in 4-hour ⁵¹Cr release assays. Interestingly, in contrast to previous reports where significant cytotoxicity was reported only after 24–48 hrs of PBL-solitomab exposure in the presence of EpCAM positive tumor cells¹², in this study we were able to demonstrate high levels of T cell cytotoxicity against chemotherapy-resistant ovarian tumor cells after only 4 hrs exposure of tumor cells with solitomab. These findings are most likely explained by the known higher sensitivity of cytotoxicity assays based on chromium release (used in our experiments) when compared to flow cytometric assays based on propidium-iodide-positive cells¹².

More importantly, we demonstrate that large amounts of ovarian tumor cells present as single cell suspension or as tumor spheroids in the ascitic fluid of ovarian cancer patients harboring advanced or chemotherapy resistant disease are also highly sensitive to solitomab-mediated cytotoxicity. The results were obtained by adding solitomab to the freshly collected tumor cells and TAL present in the ascitic fluid in the absence of any immunostimulatory cytokine and may therefore provide a preview of solitomab effects on advanced stage ovarian cancer *in vivo*, particularly when administered intra-peritoneally.

Furthermore, we have shown solitomab to be highly active at low effector: target ratios. Importantly, in addition to triggering cytotoxicity, solitomab was also capable of inducing significant CD8+ T lymphocyte proliferation, expression of activation markers and cytokine production in both autologous CD8 and CD4 TAL present in the ascitic fluid of heavily pretreated ovarian cancer patients.

For largely unknown reasons, T cell responses are blunted in later stages ovarian cancer patients¹⁸. While several reports have demonstrated that the infiltration of ovarian cancers with tumor-infiltrating lymphocytes (TILs) correlate with better survival, a large number of studies have revealed that TIL and TAL in the tumor tissues or ascitic fluid are frequently anergic and/or tolerogenic against autologous tumor cells^{18–21}. It is worth noting that in our experiments with minimally manipulated EpCAM positive tumor cells, we were able to demonstrate that all autologous TAL populations tested are able to significantly reduce the size and integrity of ovarian tumor spheroids floating in the ascitic fluid when exposed to solitomab. Taken together, these data suggest that even partially tumor-anergic T cells in ascitic fluid may become activated by solitomab and mount a cytotoxic T-cell response able to kill chemotherapy resistant ovarian cancer tumor cells.

The widespread expression and membranous localization of EpCAM in ovarian cancer cells combined with its negative expression in mesothelial type cells in the abdominal cavity, suggests that this protein could represent an accessible tumor target antigen for both intravenous and intraperitoneal antibody/BiTE®-based therapies. Consistent with this view, a phase 1 study of EpCAM/CD3-bispecific antibody (MT110) in patients harboring advanced tumors is currently ongoing²². The first clinical intraperitoneal application of a trifunctional anti-EpCAM antibody (catumaxomab/Removab®) has recently shown effective tumor cell destruction, substantially decreased ascites accumulation, and reduced necessity of paracentesis in advanced ovarian carcinoma patients harboring malignant tumors refractory to salvage chemotherapy²³. As a result, catumaxomab has recently received market approval by the European Medicines Agency (EMA) for this indication.

There is a strong need for effective novel targeted therapies in the treatment of chemotherapy-resistant ovarian cancer. Our results clearly demonstrate that solitomab may represent a novel, potentially highly effective targeted agent for the treatment of chemotherapy resistant ovarian cancer patients overexpressing Ep-CAM/TROP-1, particularly when administered in the abdominal cavity. The future design and implementation of clinical trials in this regard will ultimately determine the validity of this novel therapeutic approach.

Acknowledgments

Financial Support: This work was supported in part by grants from NIH R01 CA154460-01A1 and U01 CA176067-01A1, the Honorable Tina Brozman Foundation, the Deborah Bunn Alley Ovarian Cancer Research Foundation, and the Guido Berlucchi Research Foundation to ADS. This investigation was also supported by NIH Research Grant CA-16359 from the NCI.

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA: a cancer journal for clinicians*. 2013; 63(1):11–30. [PubMed: 23335087]
2. Bristow RE, Tomacruz RS, Armstrong DK, Trimble EL, Montz FJ. Survival effect of maximal cytoreductive surgery for advanced ovarian carcinoma during the platinum era: a meta-analysis. *J Clin Oncol*. 2002; 20(5):1248–1259. [PubMed: 11870167]
3. Bignotti E, Tassi RA, Calza S, et al. Differential gene expression profiles between tumor biopsies and short-term primary cultures of ovarian serous carcinomas: identification of novel molecular

- biomarkers for early diagnosis and therapy. *Gynecologic oncology*. 2006; 103(2):405–416. [PubMed: 16725184]
4. Santin AD, Zhan F, Bellone S, et al. Gene expression profiles in primary ovarian serous papillary tumors and normal ovarian epithelium: identification of candidate molecular markers for ovarian cancer diagnosis and therapy. *International journal of cancer Journal international du cancer*. 2004; 112(1):14–25. [PubMed: 15305371]
  5. Bellone S, Tassi R, Betti M, et al. Mammaglobin B (SCGB2A1) is a novel tumour antigen highly differentially expressed in all major histological types of ovarian cancer: implications for ovarian cancer immunotherapy. *British journal of cancer*. 2013; 109(2):462–471. [PubMed: 23807163]
  6. Armstrong A, Eck SL. EpCAM: A new therapeutic target for an old cancer antigen. *Cancer biology & therapy*. 2003; 2(4):320–326. [PubMed: 14508099]
  7. Litvinov SV, Balzar M, Winter MJ, et al. Epithelial cell adhesion molecule (Ep-CAM) modulates cell-cell interactions mediated by classic cadherins. *The Journal of cell biology*. 1997; 139(5):1337–1348. [PubMed: 9382878]
  8. Munz M, Baeuerle PA, Gires O. The emerging role of EpCAM in cancer and stem cell signaling. *Cancer research*. 2009; 69(14):5627–5629. [PubMed: 19584271]
  9. Munz M, Fellingner K, Hofmann T, Schmitt B, Gires O. Glycosylation is crucial for stability of tumour and cancer stem cell antigen EpCAM. *Front Biosci*. 2008; 13:5195–5201. [PubMed: 18508581]
  10. Spizzo G, Went P, Dirnhofer S, et al. Overexpression of epithelial cell adhesion molecule (Ep-CAM) is an independent prognostic marker for reduced survival of patients with epithelial ovarian cancer. *Gynecologic oncology*. 2006; 103(2):483–488. [PubMed: 16678891]
  11. Brischwein K, Schlereth B, Guller B, et al. MT110: a novel bispecific single-chain antibody construct with high efficacy in eradicating established tumors. *Molecular immunology*. 2006; 43(8):1129–1143. [PubMed: 16139892]
  12. Schlereth B, Fichtner I, Lorenczewski G, et al. Eradication of tumors from a human colon cancer cell line and from ovarian cancer metastases in immunodeficient mice by a single-chain Ep-CAM-/CD3-bispecific antibody construct. *Cancer research*. 2005; 65(7):2882–2889. [PubMed: 15805290]
  13. Schlereth B, Kleindienst P, Fichtner I, et al. Potent inhibition of local and disseminated tumor growth in immunocompetent mouse models by a bispecific antibody construct specific for Murine CD3. *Cancer immunology, immunotherapy : CII*. 2006; 55(7):785–796.
  14. Morecki S, Lindhofer H, Yacovlev E, Gelfand Y, Ruf P, Slavin S. Induction of long-lasting antitumor immunity by concomitant cell therapy with allogeneic lymphocytes and trifunctional bispecific antibody. *Exp Hematol*. 2008; 36(8):997–1003. [PubMed: 18495330]
  15. Morecki S, Lindhofer H, Yacovlev E, Gelfand Y, Slavin S. Use of trifunctional bispecific antibodies to prevent graft versus host disease induced by allogeneic lymphocytes. *Blood*. 2006; 107(4):1564–1569. [PubMed: 16234351]
  16. Bellone S, Siegel ER, Cocco E, et al. Overexpression of epithelial cell adhesion molecule in primary, metastatic, and recurrent/chemotherapy-resistant epithelial ovarian cancer: implications for epithelial cell adhesion molecule-specific immunotherapy. *International journal of gynecological cancer : official journal of the International Gynecological Cancer Society*. 2009; 19(5):860–866. [PubMed: 19574774]
  17. Richter CE, Cocco E, Bellone S, et al. High-grade, chemotherapy-resistant ovarian carcinomas overexpress epithelial cell adhesion molecule (EpCAM) and are highly sensitive to immunotherapy with MT201, a fully human monoclonal anti-EpCAM antibody. *American journal of obstetrics and gynecology*. 2010; 203(6):582, e581–587. [PubMed: 20870202]
  18. Costello RT, Gastaut JA, Olive D. Tumor escape from immune surveillance. *Archivum immunologiae et therapiae experimentalis*. 1999; 47(2):83–88. [PubMed: 10202560]
  19. Radoja S, Saio M, Schaer D, Koneru M, Vukmanovic S, Frey AB. CD8(+) tumor-infiltrating T cells are deficient in perforin-mediated cytolytic activity due to defective microtubule-organizing center mobilization and lytic granule exocytosis. *Journal of immunology (Baltimore, Md: 1950)*. 2001; 167(9):5042–5051.

20. Bocchia M, Bronte V, Colombo MP, et al. Antitumor vaccination: where we stand. *Haematologica*. 2000; 85(11):1172–1206. [PubMed: 11074658]
21. Sinkovics JG, Horvath JC. Vaccination against human cancers (review). *International journal of oncology*. 2000; 16(1):81–96. [PubMed: 10601552]
22. Walter, M.; Fiedler, MW.; Kebenko, Maxim; Goebeler, Marie-Elisabeth; Ritter, Barbara; Quaas, Alexander, et al. A phase I study of EpCAM/CD3-bispecific antibody (MT110) in patients with advanced solid tumors. Oral Abstract Session, Developmental Therapeutics - Clinical Pharmacology and Immunotherapy; Oral Abstract Session 2012 ASCO Annual Meeting; http://www.asco.org/ASCOv2/Meetings/Abstracts?&vmview=abst_detail_view&confID=114&abstractID=97493
23. Burges A, Wimberger P, Kumper C, et al. Effective relief of malignant ascites in patients with advanced ovarian cancer by a trifunctional anti-EpCAM x anti-CD3 antibody: a phase I/II study. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2007; 13(13):3899–3905. [PubMed: 17606723]

Figure 1.

Solitomab (EpCAM BiTE®) cytotoxicity titration graph for OSC ARK-1. Doses from 0 to 2.5 μg/ml were used in several cytotoxicity experiments aimed at determining the optimum dose of solitomab. From these experiments the 1 μg/ml concentration emerged as the optimum concentration.

Figure 2.

Graph showing antibody-dependent cell-mediated cytotoxicity for all ovarian cell lines in control conditions and with solitomab at two effector: target ratios. The higher effector: target ratio of 1:20 exhibited more pronounced cytotoxicity results.

Figure 3.

Graph showing the percentage of EpCAM positive viable cell in ascites treated for 5 days with control BiTE® vs solitomab.

Upper panel

Lower panel

Figure 4.

Upper panel Representative photograph of control BiTE treated ascites under the microscope at magnification $\times 10$. This photograph shows a Petri dish completely confluent with plastic adherent ovarian tumor cells 5 days after plating and surrounded by fewer tumor-associated lymphocytes.

Lower panel: Representative photograph of solitomab treated ascites under the microscope at magnification $\times 10$. This photograph shows a significant reduction in the number of both single ovarian tumor cells and large tumor spherocytes floating in the ascitic fluid effusates as well as ovarian cancer cells adherent and proliferating in the culture plate as compared to the control condition. Please note clusters of highly activated tumor-associated lymphocytes surrounding the few remaining viable ovarian tumor cells.

Figure 5.

CD25 and HLA-DR activation marker expression in CD8⁺ and CD4⁺ T cells after stimulation of T-lymphocytes exposed to solitomab vs control BiTE®. A significant increase in CD25 and HLA-DR activation marker expression was consistently detected in both CD8⁺ or CD4⁺ T cells.

Figure 6.

Representative IFN-gamma cytokine release after stimulation of T-lymphocytes exposed to solitomab vs control BiTE®. A significant increase in IFN-gamma stained cells was consistently detected in both CD8⁺ or CD4⁺ T cells.

Table 1

Patient characteristics and EpCAM protein expression by flow cytometry in ovarian cancer cell lines.

Cell line	Histology	Age (yrs)	Race	Stage	Grade	% EpCAM + cells	MFI	Chemotherapy
OSC ARK-1	OSC	68	W	IV	3	92.3	224.6	YES
OSC ARK-2	OSC	64	W	IIIC	3	96.4	293.6	NO
OSC ARK-3	OSC	53	W	IIIA	3	2.4	45.2	YES
A(PL)10	ACA	67	W	X	3	99.9	1129.7	YES
CC ARK-2	CC	32	W	IC	3	92.7	582.3	YES

W - White (Caucasian); OSC-Ovarian serous carcinoma, CC-Clear cell carcinoma, ACA-Mullerian ovarian adenocarcinoma. X = Advanced stage patient treated with neo-adjuvant chemotherapy.

Table 2

Patient characteristics and EpCAM protein expression by flow cytometry in ovarian cancer ascites and solid tumor component

Primary Tumor	Histology	Age (yrs)	Race	Stage	Grade	% EpCAM + cells	MFI	Chemotherapy
A(PE)49	OSC	75	W	X	3	43.0	286.1	YES
A(PE)50	OSC	85	W	IIIC	3	1.4	1280.8	YES
A(PE)51	END	46	W	IV	3	92.9 95.1*	319.0 345.3*	YES
A(PE)54	OSC	46	H	X	3	16.2	1012.9	NO
A(PE)55	OSC	59	W	IIIC	3	2.2	1226.8	YES
A(PE)57	OSC	65	W	IIIC	3	34.2	285.8	YES
A(PE)60	OSC	56	W	IIIC	3	11.8	3289.3	YES
A(PE)61	OSC	49	W	IV	3	2.2	1046.4	YES
A(PE)62	UOC	71	B	IIIC	3	12.8	220.8	NO
A(PE)64	OSC	40	W	IV	3	3.9	102.8	YES
A(PE)65	EOC	62	W	IA	1	5.6	95.6	NO
A(PE)66	OSC-CC	58	W	IIIC	3	21.2	1013.3	NO
A(PE)68	OSC	60	W	IIC	3	66.6 79.2*	2227.9 1997*	NO
A(PE)69	OSC	24	W	IIIC	2	1.9	2656.4	NO
A(PE)70	OSC	64	W	X	3	23.6	1432.2	NO
A(PE)72	MOC	66	W	IV	3	1.0	579.7	NO
A(PE)73	OSC	55	W	X	3	4.5	409.2	NO
A(PE)78	OSC	71	B	IIIC	3	6.2	4270.0	NO
A(PE)80	OSC	48	W	IIIC	3	1.5	265.7	NO
A(PE)82	OSC	84	W	IIIC	3	3.5	2935.6	YES
A(PE)83	CC	60	W	IIC	3	8.0	3868.1	NO
A(PE)84	OSC	70	W	IIIC	3	6.8	1295.6	NO
A(PE)86	OSC	62	W	IIIC	3	48	571.6	YES
A(PE)87	ACA	45	H	X	3	5.4	1439.9	YES
A(PE)88	OSC	49	W	IIIC	3	1.7	991.8	NO

Primary Tumor	Histology	Age (yrs)	Race	Stage	Grade	% EpCAM + cells	MFI	Chemotherapy
A(PE)89	OSC	82	W	IIIC	3	85	375.3	NO
A(PE)90	OSC	82	W	IIIC	3	88 91*	216.2 294.5*	NO
A(PE)91	ACA	55	W	X	3	75	223.1	YES
A(PL)8	OSC	58	W	IIIC	3	neg	neg	YES
A(PL)9	OSC	71	W	IIIC	3	neg	neg	YES
A(PL)10	ACA	67	W	X	3	50.0	195.9	YES
A(PE)95	OSC	51	W	IIIC	3	4	1268.6	NO
A(PE)97	OSC	77	W	X	3	49.5	522.3	YES
A(PE)98	OSC	81	W	IIIC	3	48.5	861.2	NO
A(PE)99	OSC	63	W	IIIC	3	20.6	2070.6	NO
A(PE)102	OSC	70	A	X	3	14.7	1321.4	YES
A(PE)103	OSC	82	W	X	3	neg	neg	YES
A(PE)106	OSC	65	W	IIIC	3	5	323.8	NO
A(PE)107	OSC	69	W	IIIC	3	neg	neg	YES
A(PE)108	OSC	35	A	IIIC	3	neg	neg	NO
A(PE)109	OSC	72	W	IIA	3	neg	neg	NO
A(PE)110	OSC	58	A	IV	3	82 85*	4539.3 3995.2*	YES

W-White (Caucasian); B-Black (African American); A-Asian; OSC-Ovarian serous carcinoma, CC-Clear cell carcinoma, UOC-Undifferentiated Ovarian Carcinoma, MOC-Mucinous ovarian carcinoma, ACA-ovarian adenocarcinoma. END – Endometrioid Ovarian carcinoma. X: Advanced stage patient treated with neo-adjuvant chemotherapy. Chemotherapy no= chemotherapy naïve tumor.

* EpCAM protein expression (% and MFI) in matched solid ovarian tumor component.