Treatment of Chemotherapy-Resistant Human Ovarian Cancer Xenografts in C.B-17/SCID Mice by Intraperitoneal Administration of Clostridium perfringens Enterotoxin

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Abstract

Ovarian cancer remains the most lethal gynecologic malignancy in the United States. Although many patients with advanced-stage disease initially respond to standard combinations of surgical and cytotoxic therapy, nearly 90% will develop recurrence and inevitably die from the development of chemotherapy-resistant disease. The discovery of novel and effective therapy against chemotherapy-resistant/recurrent ovarian cancer remains a high priority. Using expression profiling, we and others have recently found claudin-3 and claudin-4 genes to be highly expressed in ovarian cancer. Because these tight junction proteins have been described as the low- and high-affinity receptors, respectively, for the cytotoxic Clostridium perfringens enterotoxin (CPE), in this study we investigated the level of expression of claudin-3 and/or claudin-4 in chemotherapynaive and chemotherapy-resistant primary human ovarian cancers as well as their sensitivity to CPE treatment in vitro. We report that 100% (17 of 17) of the primary ovarian tumors tested overexpress one or both CPE receptors by quantitative reverse transcription-PCR. All ovarian tumors showed a dose-dependent cytotoxic effect to CPE in vitro. Importantly, chemotherapy-resistant/recurrent ovarian tumors were found to express claudin-3 and claudin-4 genes at significantly higher levels when compared with chemotherapy-naive ovarian cancers. All primary ovarian tumors tested, regardless of their resistance to chemotherapeutic agents, died within 24 hours to the exposure to 3.3 µg/mL CPE in vitro. In addition, we have studied the in vivo efficacy of i.p. CPE therapy in SCID mouse xenografts in a highly relevant clinical model of chemotherapy-resistant freshly explanted human ovarian cancer (i.e., OVA-1). Multiple i.p. administration of sublethal doses of CPE every 3 days significantly inhibited tumor growth in 100% of mice harboring 1 week established OVA-1. Repeated i.p. doses of CPE also had a significant inhibitory effect on tumor progression with extended survival of animals harboring large ovarian tumor burdens (i.e., 4-week established OVA-1).

Our findings suggest that CPE may have potential as a novel treatment for chemotherapy-resistant/recurrent ovarian cancer.

Introduction

Ovarian carcinoma remains the cancer with the highest mortality rate among gynecologic malignancies in the United States (1). Because of the insidious onset of the disease and the lack of reliable screening tests, two thirds of patients have advanced disease when diagnosed. Although many patients with disseminated tumors respond initially to standard combinations of surgical and cytotoxic therapy, nearly 90% will develop recurrence and inevitably succumb to the development of chemotherapy-resistant disease (2). The identification of novel ovarian tumor markers for early detection of the disease as well as the development of effective therapy against chemotherapy-resistant/recurrent ovarian cancer remains a high priority.

Our group as well as others have recently used high-throughput technologies, such as high-density oligonucleotide and cDNA microarrays, to analyze ovarian cancer genetic fingerprints (3–13). Among the several candidate target genes identified, genes encoding tight junction (TJ) proteins claudin-3 and claudin-4 were consistently found as two of the most highly up-regulated genes in ovarian carcinoma (3–5). Although the exact function of claudin-3 and claudin-4 overexpression in ovarian cancer is still unclear, these proteins have recently been shown to represent the natural receptors for Clostridium perfringens enterotoxin (CPE) and to be the only family members of the transmembrane tissue-specific claudin proteins capable of mediating CPE binding and cytolysis (14, 15). CPE is a single polypeptide of 35 kDa comprised of 319 amino acids that is associated with C. perfringens type A food poisoning, the second most commonly reported food-borne illness in the United States (16). CPE triggers lysis of epithelial cells through interaction with claudin-3 and claudin-4, with resultant initiation of massive permeability changes, osmotic cell ballooning, and lysis (14–16). Mammalian cells that do not express either claudin-3 and/or claudin-4 fail to bind CPE and are not susceptible to CPE cytotoxicity (16). The CPE structure/function relationship has been extensively investigated, mainly by characterizing the functional properties of enterotoxin fragments and point mutants (17–19). The CPE90-319 COOH-terminus fragment is sufficient for high affinity binding to target cell receptor and small complex formation, although this fragment is incapable of initiating large complex formation and cytolysis. Furthermore, the CPE90-319 COOH-terminus fragment inhibits cytolysis of susceptible target cells by full-length CPE. Residues 45 to 116 of CPE are essential for large complex formation and cytotoxicity, whereas deletion of the NH2-terminus generates a CPE45-319 fragment with enhanced large membrane complex formation and cytotoxic activity.

In this study, we have quantified by real-time PCR the expression levels of claudin-3 and claudin-4 receptors in several

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chemotherapy-naive and chemotherapy-resistant freshly explanted ovarian tumors. In addition, we have tested the ability of CPE to kill chemotherapy-sensitive and chemotherapy-resistant ovarian cancers overexpressing claudin-3 and/or claudin-4 in vitro. Finally, we have studied the in vitro efficacy of CPE therapy in SCID mouse xenografts in a highly relevant clinical model of chemotherapy-resistant freshly explanted human ovarian cancer (i.e., OVA-1). Here, we report for the first time that chemotherapy-resistant/ recurrent ovarian tumors highly overexpress the claudin-3 and/or claudin-4 receptors and that these chemotherapy-resistant tumors are highly sensitive to CPE treatment in vitro. More importantly, we report that in vivo therapy with repeated i.p. injections of sublethal doses of CPE induces long-term survival of the majority of animals harboring 1 week established chemotherapy-resistant ovarian disease and extended survival of animals harboring a large tumor burden of chemotherapy-resistant ovarian cancer (i.e., 4 weeks established xenografts). CPE-mediated therapy may thus represent a novel, potentially highly effective strategy for the treatment of ovarian cancer resistant to chemotherapy as well as other biologically aggressive human tumors overexpressing claudin-3 and/or claudin-4 (3, 4, 20, 21).

Materials and Methods

Cloning and purification of NH₂-terminus His-tagged Clostridium perfringens enterotoxin. C. perfringens strain 12917 obtained from American Type Culture Collection (Manassas, VA) was grown from a single colony and used to prepare bacterial DNA with the InstaGene kit according to manufacturer's directions (Bio-Rad Laboratories, Hercules, CA). The bacterial DNA fragment encoding full-length CPE gene (Genbank M90837) was PCR amplified (primer 1, 5'-GGG CAT ATG CCT GGT TAT AAAT TAA TAT TG-3'; primer 2, 5'-GAT GGA TCC TTA AAA TTT TTA GAA TAA TAT TG-3'). The PCR products were digested with the restriction enzymes NdeI/BamHI and cloned into a NdeI/BamHI–digested pET-16b expression vector (Novagen, Madison, WI) to generate an in-frame NH₂-terminus His-tagged CPE expression plasmid, pET-16b-10xHis-CPE. His- tagged CPE toxin was prepared from pET-16b-10xHis-CPE transformed Escherichia coli M15. Transformed bacteria were grown at 37°C to 0.3 or 0.4 absorbance at 600 nm, after which CPE protein expression was induced overnight with 1 mmol/L isopropyl β-D-thiogalactoside, and the cells harvested, resuspended in 150 mmol/L NaH₂PO₄ , 25 mmol/L Tris-HCL, and 8 mol/L urea (pH 8.0), and lysed by centrifugation at 10,000 rpm for 30 minutes. The fusion protein was isolated from the supernatant on a Poly-Prep Chromatography column (Bio-Rad). His-tagged CPE was washed with 300 mmol/L NaH₂PO₄, 25 mmol/L Tris-HCL, and 10 mol/L urea (pH 6.0), and eluted from the column with 200 mmol/L NaH₂PO₄, 25 mmol/L Tris-HCL and 8 mol/L urea (pH 6.0). To reduce the level of endotoxin from His-tagged CPE protein, 10 washings with ice-cold PBS with Triton X-114 (from 1% to 0.1%) and 10 washings with ice-cold PBS alone were done. Dialysis (M, 3,500 cutoff dialysis tubing) against PBS was done overnight. Purified CPE protein was then sterilized by 0.2 μm filtration and frozen in aliquots at −70°C.

Primary and established cell lines. Fresh human ovarian cancer cell lines (i.e., 11 chemotherapy-naive tumors generated from samples obtained at the time of primary surgery and six chemotherapy-resistant tumors obtained from samples collected at the time of tumor recurrence) and five established ovarian cancer cell lines (UCI 101, UCI 107, CaOV3, OVACAR-3, and OVARK-5) were evaluated for claudin-3 and claudin-4 expression by real-time PCR. Patient characteristics from which primary specimens were obtained are depicted in Table 1. Three of the six ovarian tumor specimens found resistant to chemotherapy in vivo including OVA-1, a fresh ovarian serous papillary carcinoma (OSPC) used to establish ovarian xenografts in SCID mice (i.e., severely immunocompromised animals), were confirmed to be highly resistant to multiple chemotherapeutic agents when measured as percentage cell inhibition by in vitro extreme drug resistance assay (Oncotech, Inc., Irvine, CA; ref. 22 and data not shown). UCI-101 and UCI-107, two previously characterized and established human serous ovarian cancer cell lines were kindly provided by Dr. Alberto Manetta (University of California, Irvine, CA), whereas CaOV3 and OVACAR-3 were purchased from American Type Culture Collection (Manassas, VA), and OVARK-5 was established from a stage IV ovarian cancer patient as previously described (23). Other control cell lines evaluated in the CPE assays included Vero cells, normal ovarian epithelium (NOVA), normal endometrial epithelium, normal cervical keratinocytes, primary squamous and adenocarcinoma cervical cancer cell lines, Epstein-Barr transformed B lymphocytes, and human fibroblasts. With the exception of normal cervical keratinocytes and cervical cancer cell lines that were cultured in serum-free keratinocyte medium, supplemented with 5 ng/mL epidermal growth factor and 35 to 50 μg/mL bovine pituitary extract (Invitrogen, Grand Island, NY) at 37°C, 5% CO₂, all other fresh specimens were cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (FBS; Gemini Bio-products, Calabasas, CA), 200 units/ml penicillin, and 200 μg/ml streptomycin, as previously described (3, 23, 24). All samples were obtained with appropriate consent according to Institutional Review Board guidelines. Tumors were staged according to the International Federation of Gynecology and Obstetrics operative staging system. Radical tumor debulking, including a total abdominal hysterectomy and omentectomy, was done in all ovarian carcinoma patients, whereas normal tissues was obtained from consenting similar-age donors undergoing surgery for benign pathology. Tumors were established after sterile processing of the samples from surgical biopsies as previously described for ovarian and cervical carcinoma specimens (3, 23, 24), whereas normal ovarian tissue was obtained by scraping epithelial cells from the ovarian surface. Briefly, viable tumor tissue was mechanically minced in RPMI 1640 to portions no larger than 1 to 3 mm³ and washed twice with RPMI 1640. The portions of minced tumor were then placed into 250 mL flasks containing 30 mL enzyme solution (0.1% collagenses Type I and 0.01% DNase 2,000 KU/mg Sigma) in RPMI 1640, and incubated on a magnetic stirring apparatus overnight at 4°C. Enzymatically dissociated tumor was then filtered through 150 μm nylon mesh to generate a single cell suspension. The resultant cell suspension was then washed twice in RPMI 1640 plus 10% FBS. The epithelial nature and the purity of epithelial tumor cultures was verified by immunohis-tochemical staining and flow cytometric analysis with antibodies against cytokeratin as previously described (3, 23, 24). RNA extraction was done at

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Abbreviations: OVA-R, patients with chemotherapy-resistant/recurrent disease; CC, clear cell ovarian carcinoma.

*Patients from which matched chemotherapy-naive and chemotherapy-resistant/recurrent disease were both available.

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a tumor cell confluence of 50% to 80% after a minimum of 2 to a maximum of 10 passages in vitro. Only primary cultures that had at least 90% viability and contained >99% epithelial cells were used for sensitivity to CPE in vitro.

RNA extraction and quantitative real-time PCR. RNA isolation from primary and established cell lines was done using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. Quantitative PCR was done with an ABI Prism 7000 Sequence Analyzer using the manufacturer’s recommended protocol (Applied Biosystems, Foster City, CA) to evaluate expression of claudin-3 and claudin-4 in all the samples. Each reaction was run in triplicate. Briefly, 5 μg total RNA from each sample were reverse transcribed using SuperScript III first-strand cDNA synthesis (Invitrogen, Carlsbad, CA). Five microliters of reverse transcribed RNA samples (500 μL of total volume) were amplified by using the TaqMan Universal PCR Master Mix (Applied Biosystems) to produce PCR products specific for claudin-3 and claudin-4. The primers for claudin-3 and claudin-4 were obtained from Applied Biosystems as Assay-on-Demand products. Assay IDs were Hs00265816_s1 (claudin-3) and Hs00433616_s1 (claudin-4). The comparative threshold cycle (Ct) method (PE Applied Biosystems) was used to determine gene expression in each sample relative to the value observed in the nonmalignant ovarian epithelial cells, using glyceraldehyde-3-phosphate dehydrogenase (Assay-on-Demand Hs99999905_m1) RNA as internal controls.

Claudin-4 immunostaining of formalin-fixed tumor tissues. Ovarian tumors were evaluated by standard immunohistochemical staining on formalin-fixed tissue for claudin-4 surface expression. Study blocks were selected after histopathologic review by a surgical pathologist. The most representative H&E-stained block sections were used for each specimen. Briefly, immunohistochemical stains were done on 4-μm-thick sections of formalin-fixed, paraffin-embedded tissue. After pretreatment with 10 mmol/L citrate buffer (pH 6.0) using a steamer, they were incubated with primary and established cell lines was done using TRIzol Reagent (Invitrogen, Carlsbad, CA). Antigen-bound primary antibody was detected using standard avidin–biotin immunoperoxidase complex (DAKO Corp., Carpinteria, CA). Cases with <10% staining in tumor cells were considered negative for claudin expression. The positive cases were classified as follows according to the intensity of claudin-4 protein expression: +, weak staining; ++, medium staining; and ++++, intense staining. Subcellular localization (membrane or cytoplasm) was also noted. Negative controls, in which the primary antibodies were not added, were processed in parallel.

Clostridium perfringens enterotoxin treatment of cell lines and trypan blue exclusion test. Tumor samples and normal control cells were seeded at a concentration of 1 × 10^5 cells/well into six-well culture plates (Costar, Cambridge, MA) with the appropriate medium. Adherent tumor samples, fibroblasts, and normal epithelial control cell lines were grown to 80% confluence. After washing and renewal of the medium, CPE was added to test concentrations ranging from 0.03 to 3.3 μg/mL. After incubation for 60 minutes to 24 hours at 37°C, 5% CO_2, floating cells were removed and stored, and attached cells were trypsinized and pooled with the floating cells. After staining with trypan blue, viability was determined by counting the number of trypan blue–positive cells and the total cell number.

SCID mouse tumor xenografts and Clostridium perfringens enterotoxin treatment. C.B-17/SCID female mice 5 to 7 weeks old were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and housed in a pathogen-free environment at the University of Arkansas for Medical Sciences. They were given commercial basal diet and water ad libitum. The experimental protocol for the use of these animals for these studies was approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee. Animals were used to generate ovarian tumor xenografts. The OVA-1 cancer cell line was injected i.p. at a dose of 5 × 10^6 OVA-1 tumor cells were killed at 1, 2, 3, and 4 weeks for necropsy and pathologic analysis. The remaining animals were killed and examined just before they died of i.p. carcinomatosis and malignant ascites.

Statistical differences in claudin-3 and claudin-4 expression between chemotherapy-naïve and chemotherapy-resistant ovarian tumors were tested using the Student’s t test. For the OVA-1 animal model, survivals were plotted using Kaplan-Meier methods and compared using the log-rank test. P < 0.05 was used for statistical significance.

Results

Claudin-3 and claudin-4 transcript levels in chemotherapy-sensitive and chemotherapy-resistant ovarian tumors. We used quantitative reverse transcription-PCR (qRT-PCR) assays to get highly sensitive measurements of claudin-3 and claudin-4 expression in normal tissues and fresh and established human tumors. Both claudin-3 and/or claudin-4 genes were highly expressed in all primary ovarian cancers studied when compared with normal ovarian epithelial cells as well as other normal cells or other gynecologic tumors (Fig. 1). Of interest, established ovarian cancer cell lines (UCI 101, UCI 107, CaOV3, OVACAR-3, and OVARK-5) were found to express much lower levels of claudin-3 and/or claudin-4 compared with primary ovarian tumors (Fig. 1). Finally, claudin-3 and/or claudin-4 expression was extremely low in all control tissues examined, including normal ovarian epithelium, normal endometrial epithelium, normal cervical keratinocytes, and normal human fibroblasts (Fig. 1).
When OSPC collected at the time of primary debulking surgery (six cases) were compared for claudin-3 and/or claudin-4 receptor expression to those collected at the time of tumor recurrence after multiple courses of chemotherapy (six cases), chemotherapy-resistant tumors were found to express significantly higher levels of claudin-3 and/or claudin-4 receptors ($P < 0.05$; Fig. 2). Importantly, when three primary ovarian cancers naïve to chemotherapy were compared with recurrent ovarian cancers recovered from the same patients following chemotherapy (i.e., matched autologous tumor samples), chemotherapy-resistant tumors were again found to express higher levels of claudin-3 and claudin-4 (Fig. 2).

Claudin-4 expression by immunohistology on ovarian serous papillary carcinoma and normal ovarian epithelium tissue blocks. To determine whether the high expression of the claudin-4 gene detected by qRT-PCR assays in primary ovarian cancer cell lines is the result of a selection of a subpopulation of cancer cells present in the original tumor or whether in vitro expansion conditions may have modified gene expression, we did immunohistochemical analysis of claudin-4 protein expression on formalin-fixed tumor tissue from the uncultured primary surgical specimens from which fresh ovarian cancers were derived. As shown in Table 2 and representatively in Fig. 3 (right), moderate to heavy membranous staining for claudin-4 protein expression was noted in all the cancer specimens that overexpressed the claudin-4 transcript. In contrast, negative or low staining was found in all the normal ovarian epithelium tested by immunohistochemistry (Fig. 3; Table 2).

Effects of Clostridium perfringens enterotoxin on fresh ovarian and cervical cancer cell lines and normal control cells. On the basis of the high expression of claudin-3 and/or claudin-4 on primary ovarian cancer cell lines previously reported by us in a small group of patients (5), it was expected that ovarian tumors expressing either claudin-3 or claudin-4 would be sensitive to CPE-mediated lysis. However, it was important to show this directly on fresh human ovarian carcinoma cells, particularly in a clinically relevant setting of ovarian cancer disease for which current salvage therapies are ineffective (i.e., chemotherapy-resistant disease). For this reason, we examined short-term in vitro primary cultures of ovarian carcinomas obtained either from chemotherapy-naïve patients (i.e., OVA-2, OVA-3, and OVA-5) or patients heavily treated with different combinations of chemotherapy (i.e., OVA-1, OVA-4, and OVA-6) and now with disease progression after multiple chemotherapy regimens. The

Table 2. Claudin-4 staining

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sensitivity of these primary ovarian tumor cultures to CPE-mediated cytolysis was tested along with an appropriate claudin-3 and claudin-4–expressing positive control (i.e., Vero cells), established OSPC cell lines (OVARK-5, CaOV3, and OVACAR-3), and negative controls that do not express detectable levels of either claudin-3 or claudin-4. As shown in Fig. 4, regardless of their sensitivity or resistance to chemotherapy, all ovarian tumors tested were found sensitive to CPE-mediated cytolysis. The cytotoxic effect was dose dependent and was positively correlated to the levels of either claudin-3 or claudin-4 expression as tested by RT-PCR in tumor samples. Importantly, although ovarian tumors showed different sensitivities to CPE exposure, no ovarian cancer was found viable after 24 hours exposure to CPE at the concentration of 3.3 μg/mL. In contrast, all normal controls tested
including ovarian epithelium, cervical keratinocytes, and mono-
nuclear cells as well as cervical cancer cell lines lacking claudin-3
or claudin-4 were not affected by CPE (Fig. 4).

**Effect of Clostridium perfringens enterotoxin on chemotherapyresistant ovarian cancer cells in vivo.** For in vitro confirmation of
our in vitro data, we have developed xenograft tumors in SCID mice
by i.p. injection with OVA-1, a primary ovarian tumor resistant to
multiple chemotherapeutic agents in vitro (by extreme drug
resistance assay) as well as in vivo. Primary OVA-1 tumor cells grew
progressively as numerous serosal nodules adherent to virtually all intraabdominal organs (peritoneum, omentum, diaphragm, bowel,
liver, pancreas, and spleen) and exhibited the capacity for local tissue
invasion and formation of malignant ascites after 2 to 3 weeks from
injection. Tumors became evident by the second week as small
nodules on the omentum and continuously grew to form a confluent
omental mass by the time the animals died (i.e., mean survival 38
days after i.p. injection with 7.5 × 10⁶ OVA-1 cells; Fig. 5). Necropsies
revealed massive hemorrhagic ascites and numerous tumor nodules,
measuring 1 to 8 mm in diameter, studding the entire peritoneal
surface and implanting the serosa of virtually all intraabdominal
organs (Fig. 5).

Previous toxicology studies in mice have reported 0.5 μg/g
CPE administered i.p. to be a well tolerated and safe dose in
100% of the animals (i.e., 16.5 ± 1.0 g male SW mice; refs. 25,
26). In contrast, some animals injected with 0.75 μg/g died after
CPE injection and all animals injected with 1 μg/g of CPE died
within 1 to 2 hours (25, 26). Our determination of maximum
tolerated dose in healthy female mice is consistent with these
observations (data not shown). In one experiment, groups of
mice harboring large ovarian tumor burden xenografts (i.e.,
4 weeks after OVA-1 tumor injection) were treated with repeated
i.p. CPE injections every 72 hours at three different doses (5.0,
5.5, or 6.5 μg). Control mice harboring OVA-1 received saline
alone. CPE injections were well tolerated and no adverse events
were observed throughout the complete treatment protocol
either in control mice receiving CPE alone or CPE-treated mice
harboring large tumor burden. Mice harboring OVA-1 treated
with saline all died within 6 weeks from tumor injection with a
mean survival of 38 days (Fig. 6A). In contrast, animals treated
with multiple CPE injections survived significantly longer than
treatment animals did (P < 0.0001; Fig. 6A). The increase in
survival in the different groups of mice treated with the diverse
doses of CPE was clearly dose dependent, with the highest dose
injected (i.e., 6.5 μg every 72 hours) found to provide the longer
survival (Fig. 6A). In another set of experiments, mice harboring
OVA-1 (a week after tumor injection with 5 × 10⁶ cells) were
injected with i.p. CPE injections at a dose ranging from 7.5 to
8.5 μg every 72 hours. Whereas mice harboring OVA-1 treated
with saline all died within 9 weeks from tumor injection (Fig.
6B), three of five (60%) and five of five (100%) of the mice
injected with multiple i.p. injections of CPE remained alive and
free of detectable tumor for the duration of the study period
(i.e., over 120 days, P < 0.0001).

**Discussion**

Several groups, including our own, have recently shown by gene
expression profiling that claudin-3 and claudin-4 are highly
overexpressed in primary ovarian carcinomas (3–5), the most
gynecologic malignancy in the United States. These findings
imply that ovarian cancer refractory to standard treatment
modalities may be susceptible to CPE-based therapeutic
approaches. In this study, we have confirmed the high expression
of CPE receptors at both the RNA and protein levels in multiple
primary ovarian cancers and tested the sensitivity of tumor cells
to CPE treatment in vitro. Pharmacologic studies in ovarian cancer
patients have shown a marked therapeutic advantage to the i.p.
delivery of drugs and biologicals combined with a significant
reduction in systemic toxicity resulting from i.p. drug administra-
tion when compared with an identical dose of the drug given i.v.
(27). These clinical observations, combined with the fact that
ovarian cancer remains confined to the peritoneal cavity for much
of its natural history, suggest that i.p. administration of CPE in
human patients harboring recurrent ovarian cancer refractory to
Chemotherapy may result in reduced toxicity and better therapeutic responses compared with an identical dose of CPE given i.v. Accordingly, we have also conducted a careful evaluation of the efficacy and toxicity of i.p. injection of CPE in vivo in a clinically relevant animal model of chemotherapy-resistant ovarian tumor xenografts.

Our studies showed that 100% (17 of 17) of the primary ovarian cancer cell lines tested for claudin-3 and claudin-4 expression by qRT-PCR overexpress either the high-affinity CPE receptor (claudin-4) or the low-affinity CPE receptor (claudin-3). Of interest, all the established ovarian cancer cell lines tested (UCI 101, UCI 107, CaOV3, OVACAR-3, and OVARK-5) were found to express much lower levels of claudin-3 and/or claudin-4 compared with primary ovarian tumors. These data suggest that prolonged in vitro culture may significantly alter claudin-3 and claudin-4 gene expression in ovarian cancer. In addition, we have noticed a consistent down-regulation of claudin-3 and claudin-4 expression levels by qRT-PCR in the more advanced in vitro passages of primary OSPC (data not shown). Thus, established ovarian cancer cell lines may represent suboptimal models to evaluate the potential of CPE-mediated therapy against ovarian cancer in vitro as well as in vivo. Importantly, all primary ovarian tumors evaluated, including those found to be resistant to chemotherapy in vivo as well as in vitro, were found highly sensitive to CPE-mediated killing in vitro. In this regard, although ovarian tumors showed different sensitivity to CPE exposure, no ovarian cancer was found viable after 24 hours exposure to CPE at the concentration of 3.3 μg/mL, a dose well tolerated by i.p. administration of CPE in our animal model. This was in strong contrast with the lack of sensitivity of normal ovarian epithelium as well as other normal control cells to CPE-mediated cytolysis. These findings are likely explained by a limited expression of claudin-3 and claudin-4 in normal epithelia compared with ovarian tumor cells.

When the efficacy of multiple i.p. injection of sublethal doses of CPE in vivo in a clinically relevant animal model of chemotherapy-resistant ovarian tumor xenografts was tested, we found that doses of CPE ranging from 5 to 8.5 μg/mL were well tolerated, and
no adverse events were observed throughout the complete treatment protocol either in control mice receiving CPE alone or CPE-treated mice harboring small and large tumor burden. These data show that CPE doses found effective in vitro to kill ovarian tumor cells may be safely administered i.p. in mice harboring ovarian cancer disease. More importantly, we found that survival of mice harboring a large burden of chemotherap Resistant ovarian disease was significantly prolonged in a dose-dependent manner by repeated i.p. injections of CPE. Finally, when animals harboring 1 week OVA-1 xenografts were treated with repeated i.p. injections of CPE, most of the mice remained alive and free of detectable tumor for the duration of the study (i.e., over 120 days). Collectively, these results provide strong evidence to suggest that CPE-based therapy may have great potential in the treatment of ovarian cancer patients refractory to standard treatment modalities.

Despite the ability of CPE to effectively lyse chemotherapy-resistant ovarian cancers in vitro as well as in vivo, the local delivery of CPE for its clinical application in human patients faces several limitations. Indeed, as with any foreign protein, multiple administration of CPE may likely induce the development of neutralizing antibodies in ovarian cancer patients, which may prevent or reduce the efficacy of repeated CPE administrations. Thus, although it has been previously observed that local defects in immune responses are commonly detected in the peritoneal cavity of advanced-stage ovarian cancer patients (28), and also that elevated titers of antianti- toxin antibodies that arise following CPE ingestion provide no protection for human subjects against the effects of subsequent ingestion of CPE (29), careful studies need to be done to accurately determine the presence and nature of the immune response against CPE when administered by the i.p. route in human patients.

Another potential problem with the i.p. administration of CPE in ovarian cancer patients is the necessity for the toxin to distribute evenly throughout the abdominal cavity to properly reach the tumor tissue. This point is noteworthy because previous surgery and subsequent adhesions, which can prevent homogenous fluid distribution, might greatly reduce the efficacy of local CPE therapy. In addition, because i.p. ovarian tumor plaques will likely receive CPE by passive diffusion (i.e., typically only a few millimeters), the inability of local CPE administration to deeply penetrate large solid tumor masses may also reduce the efficacy of local CPE therapy in patients harboring a large tumor burden. Taken together, these points imply that local CPE administration would likely exert its maximum benefit in patients with microscopic residual disease or small-volume macroscopic cancer resistant to standard chemotherapeutic agents.

Although the clinical application of CPE faces several challenges, it has also several potential advantages. Indeed, CPE-mediated cytolysis requires only the single step of CPE binding to its receptors and take place after only few minutes of tumor cell exposure to toxic CPE concentrations. Thus, the simplicity and rapidity of CPE-mediated cytolysis may result in increased efficacy, reduced opportunity for the development of resistance, and the possibility that high local concentrations of CPE may need to be maintained for only a relatively short period of time. Furthermore, because the efficacy of CPE therapy against chemotherapy-resistant ovarian cancer xenografts has been shown in SCID mice, it seems unlikely that the host immune system is required to play a significant role in the in vivo efficacy of CPE therapy. This point is noteworthy because several biological response modifiers previously used for the therapy of chemotherapy-resistant ovarian cancer, including cytokines (30) and humanized monoclonal antibodies (31), unlike CPE therapy, rely for most of their efficacy on the activation of an uncompromised host immune system (32), a major limitation when dealing with elderly ovarian cancer patients heavily pretreated with multiple regimes of immunosuppressive chemotherapy.

In conclusion, we have shown that primary ovarian cancers that have acquired in vivo resistance to chemotherapeutic drugs may be susceptible to killing by CPE-mediated therapy in vitro as well as in vivo. Taken altogether, our findings suggest that i.p. CPE administration may have potential as a novel strategy against chemotherapy-resistant/recurrent ovarian cancer. The future design and implementation of phase I and phase II clinical trials in patients harboring chemotherapy refractory ovarian disease will determine the feasibility and validity of this novel therapeutic approach.

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