Overexpression of Clostridium perfringens Enterotoxin Receptors Claudin-3 and Claudin-4 in Uterine Carcinosarcomas

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Abstract

Purpose: To evaluate the expression levels of claudin-3 and claudin-4, the low- and high-affinity receptors, respectively, for the cytotoxic Clostridium perfringens enterotoxin (CPE) in uterine carcinosarcomas and explore the potential for targeting these receptors in the treatment of this aggressive uterine tumor.

Experimental Design: We analyzed claudin-3 and claudin-4 receptor expression at mRNA and protein levels in flash frozen and formalin-fixed, paraffin-embedded carcinosarcoma specimens. Recombinant CPE was used as a novel therapy against chemotherapy-resistant carcinosarcoma cell lines in vitro. The therapeutic effect of sublethal doses of CPE was studied in severe combined immunodeficient mouse xenografts harboring large s.c. carcinosarcomas.

Results: All flash-frozen carcinosarcoma biopsies (12 of 12) and short-term carcinosarcoma cell lines evaluated overexpressed claudin-3 and claudin-4 by quantitative reverse transcription-PCR. Membranous immunoreactivity for claudin-4 protein expression was documented in 80% (20 of 25) of primary tumors and 100% (6 of 6) of the metastatic carcinosarcomas, whereas negligible staining was found in normal endometrial cells. Regardless of their resistance to chemotherapeutic agents, all short-term carcinosarcoma cell lines tested died within 1 h of exposure to 3.3 μg/mL of CPE in vitro. Intratumoral injections of well-tolerated doses of CPE in large s.c. carcinosarcoma xenografts led to large areas of tumor necrosis and tumor disappearance in all treated animals.

Conclusions: Claudin-3 and claudin-4 receptors are highly overexpressed in carcinosarcoma. These proteins may offer promising targets for the use of CPE as a novel type-specific therapy against this biologically aggressive variant of endometrial cancer.

Uterine carcinosarcomas are highly aggressive malignancies containing both carcinomatous and sarcomatous elements. They represent the most common of the uterine sarcomas accounting for 1,000 to 1,500 cases annually in the United States (1). They usually arise in elderly postmenopausal women and, because of their aggressive biological behavior and early spreading pattern, are often diagnosed at advanced stage. Carcinosarcomas are characterized by a poor prognosis with overall 5-year survival rates that range from 33% to 39% (2).

The identification of novel molecular markers to be used as targets for innovative, potentially effective therapies against this aggressive variant of uterine sarcoma remains a high priority.

Using gene expression profiling, claudin-3 and claudin-4 genes have been recently reported by our group to be highly differentially expressed in several biologically aggressive gynecologic malignancies, including ovarian serous papillary carcinoma and uterine serous papillary carcinoma (3, 4). Importantly, claudin-3 and claudin-4 tight junction proteins have 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 (5, 6). CPE is a single polypeptide of 35 kDa composed 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 (7). CPE triggers lysis of epithelial cells through interaction with claudin-3 and claudin-4 receptors with resultant collapse of the cellular colloid-osmotic equilibrium and initiation of massive permeability changes leading to osmotic cell ballooning and lysis (5–7). Mammalian cells that do not express either claudin-3 or claudin-4 fail to bind CPE and are not susceptible to CPE cytotoxicity (7–10).

In this study, we have quantified the expression levels of claudin-3 and claudin-4 receptors by real-time PCR in several...
freshly explanted carcinosarcomas. In addition, we analyzed claudin-4 receptor expression at the protein level by immunohistochemistry in a separate set of formalin-fixed, paraffin-embedded primary and metastatic carcinosarcomas. Finally, we have tested the ability of recombinant CPE to kill chemotherapy-resistant carcinosarcoma cell lines in vitro as well as in vivo. Here, we report the first evidence that carcinosarcomas highly overexpress the claudin-3 and claudin-4 receptors and that these biologically aggressive tumors, regardless of their resistance to chemotherapy, are highly sensitive to CPE treatment in vitro as well as in vivo.

Materials and Methods

Cloning and purification of NH2-terminus His-tagged CPE. C. perfringens strain 12917 obtained from American Type Culture Collection was grown from a single colony and used to prepare bacterial DNA with the InstaGene matrix, according to the manufacturer’s directions (Bio-Rad). The bacterial DNA fragment encoding full-length CPE gene (Genbank accession no. A000766) was PCR amplified (primer 1, 5’-AGATGTATAAGCTGATTCATGTAACCAATTTGAAAGG-3’; primer 2, 5’-AAAAAGGTTTTAATTTTTGCAAATAATTTGAAAGGGG-3’). The PCR products were digested with the restriction enzymes NdeI/BamHI and cloned into a NdeI/BamHI-digested pET-16b expression vector (Novagen) to generate an in-frame NH2-terminus His-tagged CPE expression plasmid, pET-16b-10. His-tagged CPE toxin was prepared from pET-16b-10×His-CPE transformed Escherichia coli M15. Transformed bacteria were grown at 37°C to 0.3 to 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 were harvested, resuspended in 150 mmol/L NaH2PO4, 25 mmol/L Tris-HCl, and 8 mol/L urea (pH 8.0) buffer, and lysed by centrifugation at 10,000 rpm for 30 min. 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.

Flash frozen biopsies and primary cell lines. Twelve flash-frozen biopsy specimens containing >75% neoplastic cells obtained from carcinosarcoma patients at the time of primary surgery, and three short-term carcinosarcoma cell lines (two generated from tumor samples from primary uterine site and one from metastatic intra-abdominal disease), 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. One of the three carcinosarcoma specimens established as short-term cultures (i.e., SARC-1) was confirmed to be highly resistant to multiple chemotherapeutic agents when measured as percentage cell inhibition by in vitro extreme drug resistance assay (Oncotech; data not shown). Control cell lines evaluated in the CPE assays included Vero cells (purchased from American Type Culture Collection), normal endometrial cells (NEC), normal cervical keratinocytes, peripheral blood lymphocytes, EBV-transformed B lymphocytes, and human fibroblasts. With the exception of normal cervical keratinocytes, which 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) at 37°C; 5% CO2, all other fresh specimens were cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (Gemini Bio-Products), 200 units/mL penicillin, and 200 μg/mL streptomycin. All samples were prepared from patients with appropriate consent according to institutional review board guidelines. Tumors were staged according to the International Federation of Gynecology and Obstetrics operative staging system. Tumor debulking included a total abdominal hysterectomy with bilateral salpingo-oophorectomy and bilateral pelvic lymphadenectomy in all carcinosarcoma patients, whereas normal ovaries were obtained from consenting donors undergoing surgery for benign pathology. Tumors and normal primary control tissues were established after sterile processing of the samples from surgical biopsies as previously described (3, 4). 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 of enzyme solution (0.14% collagenase type I and 0.01% DNase; 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% fetal bovine serum. The epithelial nature and the purity of epithelial tumor cultures was verified by immunohistochemical staining and flow cytometric analysis with antibodies against cytokeratin as previously described (3, 4). In carcinosarcoma cell lines, RNA extraction was done at a tumor cell confluence of 50% to 80%. Only primary cultures that had at least 90% viability and contained >95% tumor cells were used to test sensitivity to CPE in vitro.

RNA extraction and quantitative real-time PCR. RNA isolation from flash-frozen and primary 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) to evaluate expression of claudin-3 and claudin-4 in all the samples. Each reaction was run in triplicate. Briefly, 1 μg of total RNA from each sample was reverse transcribed using SuperScript III first-strand cDNA synthesis (Invitrogen). Five microliters of reverse transcribed RNA samples (from 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 in all the samples. 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 Hs00533616_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 lowest nonmalignant endometrial epithelial cell sample, using

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*Patients from which primary carcinosarcoma short-term cell lines were established to be used for in vitro and in vivo CPE sensitivity studies.

†Patient from which a metastatic carcinosarcoma short-term cell line was established to be used for in vitro and in vivo CPE sensitivity studies.
glyceraldehyde-3-phosphate dehydrogenase (Assay-on-Demand Hs99999905_m1) RNA as internal controls.

**Claudin-4 immunostaining of formalin-fixed tumor tissues.** A total of 25 primary, six metastatic carcinosarcomas, and four NEC control tissues obtained from similar-age postmenopausal women were evaluated by standard immunohistochemical staining on formalin-fixed tumor tissue for claudin-4 surface expression. Study blocks were selected after histopathologic review by a surgical pathologist (I.K.M.). The most representative block was selected for each specimen, and each included both the epithelial and mesenchymal components of the neoplasm. 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 mouse anti–claudin-4 antibodies (Zymed Laboratories, Inc.). Antigen-bound primary antibody was detected using standard avidin-biotin immunoperoxidase complex (DAKO Corp.). Cases with <10% membranous staining in tumor cells were considered negative for claudin expression. The intensity of membranous immunoreactivity for claudin-4 in tumor cells was subjectively scored as follows: (a) 0, negative; (b) 1+, weak staining; (c) 2+, medium staining; and (d) 3+, intense staining. Negative controls, in which the primary antibodies were not added, were processed in parallel. The pathologist was unaware of which slides were stained with isotype or claudin-4 antibodies.

**CPE treatment of cell lines and trypan blue exclusion test.** Tumor samples and normal control cells were seeded at a concentration of 1 × 10⁵ per well into six-well culture plates (Coster) 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 final concentrations ranging from 0.03 to 3.3 μg/mL. After incubation for 60 min to 24 h at 37°C, 5% CO₂, 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.

**Severe combined immunodeficient mouse tumor xenografts and CPE treatment.** C.B-17/severe combined immunodeficient (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 s.c. carcinosarcoma xenografts. Briefly, 10 × 10⁵ SARC-1 cells in 0.1 mL normal saline were injected s.c. into the left flank in groups of three to five mice. Twelve weeks after SARC-1 injection, when tumor size was ~2 cm in diameter, 1 μg CPE dissolved in 100 μL 0.9% NaCl or saline alone was injected intratumorally daily in the large tumor area for five consecutive days for 2 weeks. On day 15, some mice were euthanized to determine tumor size, whereas others were followed to evaluate survival and possible tumor reappearance. Explanted tumors were stored in 2% formaldehyde and stained with H&E for histologic examination. Each SCID mouse experiment was done in duplicate.

**Statistics.** Flash-frozen specimens and short-term cultures of carcinosarcoma (SARC) were compared with NEC for claudin-3 and claudin-4 differences in quantitative reverse transcription-PCR (qRTPCR) expression via Wilcoxon rank-sum (WRS) test with normal approximation. The WRS test with same approximation was also used to compare primary SARC tissue to NEC tissue for differences in intensity of immunohistochemical staining for claudin-4. To assess claudin-3 and claudin-4 expression trends over time in short-term SARC cell lines, log₂-transformed expression ratios were regressed on the rank order of in vitro passages, and the regression slopes wereback-transformed to yield average fold decreases in copy number per step. P values <0.05 were considered statistically significant.

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**Results**

Claudin-3 and claudin-4 transcript levels in carcinosarcomas. We used qRT-PCR assays to get highly sensitive measurements of claudin-3 and claudin-4 expression in 12 flash-frozen carcinosarcoma specimens, three short-term carcinosarcoma cultures, and several flash-frozen normal tissues and cell lines. Figure 1 shows expression details for SARC versus NEC. Both claudin-3 and claudin-4 genes were highly expressed in all flash-frozen carcinosarcoma specimens when compared with NEC (Fig. 1). Normal cervical keratinocytes, EBV-transformed B cells, and human fibroblasts expressed low levels of CPE receptors. Indeed, in cervical keratinocytes claudin-3 had a median (minimum-maximum) mRNA copy number of 25 (3-89), whereas claudin-4 had a median (minimum-maximum) mRNA copy number of 31.5 (13-57). In EBV-transformed B lymphocytes, claudin-3 had a median (minimum-maximum) mRNA copy number of 21 (2-69), whereas claudin-4 had a median (minimum-maximum) mRNA copy number of 27 (11-47). Similarly, in normal fibroblasts, claudin-3 had a median (minimum-maximum) mRNA copy number of 15 (2-49), whereas claudin-4 had a median (minimum-maximum) mRNA copy number of 21.5 (3-58). In contrast, in the carcinosarcoma flash-frozen samples, claudin-3 had a median (minimum-maximum) copy number of 556.5 (132-1,381), compared with 12.5 (1-119) in the NEC (WRS |Z| = 2.91; P = 0.004). Claudin-4 had a median (minimum-maximum) copy number of 311.5 (139-3,057) in the samples, compared with 18.5 (1-80) in the NEC (WRS |Z| = 2.91; P = 0.004). Note that for both claudin mRNAs, the maximum copy number in the NEC was less than the minimum copy number in the flash-frozen samples. When three short-term carcinosarcoma cultures (i.e., SARC-1, SARC-2, and SARC-3) were likewise compared with the NEC (Fig. 1), they showed the same elevations of expression and same nonoverlap of ranges: Claudin-3 had a median (minimum-maximum) copy number of 1,380 (380-1,863) in the short-term cultures, compared with 12.5 (1-119) in the NEC (WRS |Z| = 2.12; P = 0.034), whereas claudin-4 had a median (minimum-maximum) copy number of 710 (655-783) in the cultures, compared with 18.5 (1-80) in the NEC (WRS |Z| = 2.12; P = 0.034). Importantly, as representatively shown in Fig. 2 for SARC-1 and SARC-2 cell lines, when claudin-3 and claudin-4 gene expression levels were tested via regression for trend over multiple time points after prolonged in vitro culture, we observed a consistent downward trend in expression levels of both the CPE receptors in the more advanced in vitro passages by qRT-PCR. The average fold decrease (95% confidence interval) from bar to bar in each bar chart (Fig. 2) was as follows: 1.29x (1.24x-1.34x) for claudin-3 in SARC-1 (regression r² = 99.8%, F₂,₁ = 874.7; P = 0.001), 2.14x (1.60x-2.85x) for claudin-4 in SARC-1 (regression r² = 98.5%, F₂,₁ = 129.3; P = 0.008), 2.56x (1.15x-5.69x) for claudin-3 in SARC-2 (regression r² = 92.7%, F₂,₁ = 25.6; P = 0.037), and 2.92x (1.82x-4.71x) for claudin-4 in SARC-2 (regression r² = 97.9%, F₂,₁ = 93.8; P = 0.011). Based on these results, cell lines at early passages were used as models to evaluate the potential of CPE-mediated therapy against carcinosarcomas in vitro as well as in vivo (see below).

Claudin-4 expression by immunohistochemistry on carcinosarcoma and NEC tissue blocks. To determine whether the high expression of claudin-3 and claudin-4 genes detected by
qRT-PCR assays in primary carcinosarcomas may result in high surface expression of the CPE receptors on tumor cells, we did immunohistochemical analysis of claudin-4 protein expression on formalin-fixed tumor tissue from a separate set of 25 formalin-fixed, paraffin-embedded carcinosarcoma specimens. Both the epithelial and sarcomatous component of the tumors were evaluated for claudin-4 expression. As shown in Table 2 and representatively in Fig. 3, the intensity of staining for claudin-4 was significantly higher \((WRS |Z| = 2.63; P = 0.009)\) among the 25 carcinosarcoma specimens compared with the four NEC. With no exception, all immunohistochemistry-positive tumors showed membranous positivity for claudin-4 in the majority (i.e., mean 54%) of the epithelial cell component of the carcinosarcomas (Fig. 3). Indeed, only 2 of the 25 tumors were found to have a low positivity (1+) for the claudin-4 receptor in <10% of the sarcomatous (i.e., spindle-cell) component of the tumor, whereas the remaining 23 tumors showed no claudin-4 staining in the sarcomatous cells. Metastatic tumor sites were also available for immunohistochemical testing in six of the patients found to overexpress claudin-4 in the primary tumor. All six of the carcinosarcoma metastatic specimens tested by immunohistochemistry were found to overexpress claudin-4 at moderate to high levels (i.e., 2+ and 3+) in the epithelial cell component of the tumor (Table 3).

**Effects of CPE on primary and metastatic carcinosarcoma and normal control cells.** We examined and tested the sensitivity of short-term *in vitro* cultures of carcinosarcoma obtained either from the primary endometrial site (i.e., SARC-1 and SARC-2) or from metastatic locations (SARC-3) to scalar doses of CPE. The level of endotoxin in the CPE preparations used in *in vitro* and *in vivo* experiments ever exceed 1 endotoxin unit per milliliter by Limulus amebocyte assay. The sensitivity of these primary carcinosarcoma cultures to CPE-mediated cytolysis was tested along with an appropriate claudin-3– and claudin-4–expressing positive control (i.e., Vero cells) and negative controls not expressing either claudin. As shown in Fig. 4, all three carcinosarcomas tested, regardless of their primary or metastatic...
origin or their resistance to chemotherapy (i.e., SARC-1), were highly sensitive to CPE-mediated cytolysis. In contrast, all normal control cells tested, including NEC, cervical keratinocytes, human fibroblasts, and mononuclear cells lacking claudin-3 or claudin-4 expression, were not affected by CPE (Fig. 4).

**Effect of CPE on xenografted chemotherapy resistant carcinosarcoma cells in vivo.** We established s.c. carcinosarcoma xenografts in SCID mice by injection of claudin-3– and claudin-4–expressing SARC-1, a highly chemotherapy-resistant carcinosarcoma cell line. After the formation of large s.c. tumors (i.e., 2 cm in diameter), animals were treated with a total of 10 injections of CPE or saline directly into the tumor over a 2-week period. As representatively shown in Fig. 5, s.c. CPE-treated SARC-1 tumors showed a dramatic reduction in tumor size, with clinical disappearance of the disease in all CPE-treated animals. In contrast, animals treated with saline control injections developed rapidly progressing disease and had to be euthanized 14 to 16 weeks from the beginning of the experiment. All animals examined for histologic confirmation of regression after CPE treatment showed large necrotic areas (Fig. 6). In contrast, we found no necrosis in the histologic sections of all control animals injected with saline (not shown).

### Discussion

Endometrial carcinosarcomas are biologically highly aggressive tumors (1, 2). There is still considerable debate as to whether carcinosarcomas should be classified as sarcomas or as

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<td>5 (20%)</td>
<td>6 (24%)</td>
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**NOTE:** Data are number (%) at each intensity score (see Table 3).

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<td>Claudin-4 staining in primary carcinosarcomas</td>
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<tr>
<td>NEC 4 (100%)</td>
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<tr>
<td>SARC 5 (20%)</td>
<td>5 (20%)</td>
<td>6 (24%)</td>
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WRS test for group difference in immunohistochemical scores: $|Z| = 2.63, P = 0.009$.  

![Fig. 3. Representative immunohistochemical staining for claudin-4 on NEC paraffin-embedded specimen (A), a primary carcinosarcoma specimen (B and C), and a metastatic carcinosarcoma specimen (D). NEC1 and the spindle tumor cell component of the primary carcinosarcoma showed light (A) to negligible (B) membrane staining for claudin-4, whereas the epithelial component of the primary (C) and metastatic (D) carcinosarcoma showed strong cytoplasmic and membranous reactivity for claudin-4. Original magnification, ×400.](image-url)
carcinomas with sarcomatous differentiation (11). Indeed, there is evidence that most of these tumors are monoclonal in origin and that the carcinomatous component represents the true driving force of the disease (11). Regardless of their classification, however, the overall prognosis of patients diagnosed with this uncommon disease remains extremely poor (1, 2, 11). Our group has recently evaluated the genetic fingerprint of high-grade serous papillary uterine and ovarian carcinomas (3, 4).

Claudin-3 and claudin-4, the natural receptors for CPE, were found among the highest differentially expressed genes when compared with normal ovarian or endometrial control cells, respectively (3, 4).

In the last few years, the CPE structure/function relationship has been extensively investigated, mainly by characterizing the functional properties of enterotoxin fragments and point mutations.
mutants (8–10). The CPE290-319 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 CPE290-319 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 carefully evaluated the expression of CPE receptors at both RNA and protein levels in multiple flash-frozen carcinosarcoma biopsies and primary carcinosarcoma cell lines. In addition, we have studied the sensitivity of primary, metastatic, and chemotherapy-resistant carcinosarcoma cell lines to CPE treatment in vitro. Finally, we have tested the in vivo efficacy of local CPE administrations as novel therapy in SCID mouse xenograft models harboring established carcinosarcomas refractory to chemotherapy.

Our studies showed that 100% (12 of 12) of the carcinosarcomas tested for claudin-3 and claudin-4 expression by qRT-PCR overexpress both the high-affinity CPE receptor (claudin-4) and the low-affinity CPE receptor (claudin-3). Of interest, overexpression of claudin-3 and claudin-4 has recently been associated with an increase in tumor cell motility, invasion capability, and tumor cell survival in ovarian carcinomas (12). These recent data, combined with the high expression of claudin-3 and claudin-4 found in our study, further support the concept that overexpression of the CPE receptors may represent a marker of biological aggressiveness that correlates with the metastatic process. Indeed, when immunohistochemistry was used to confirm high expression of claudin-4 protein on primary and metastatic carcinosarcoma, we consistently found high expression in the epithelial but not in the sarcomatous component of the carcinosarcomas.

Importantly, the extremely high expression of claudin-3 and claudin-4 detected in all metastatic SARC cell lines evaluated thus far by qRT-PCR, including SARC-1, a highly chemotherapy resistant tumor cell line, suggested that biologically aggressive carcinosarcomas may be particularly susceptible to CPE-mediated killing in vitro as well as in vivo. In agreement with this view, with no exception, all three carcinosarcoma cell lines available to this study were found not viable within 24 h exposure to CPE at the concentration of 3.3 µg/mL, a dose well-tolerated when administered i.p. in animal models harboring chemotherapy-resistant ovarian serous papillary carcinoma xenografts overexpressing claudin-3 and claudin-4 receptors (3). Moreover, in all carcinosarcoma cell lines, we found that exposure to CPE doses as low as 0.8 µg/mL was able to induce massive “bleb ballooning” and tumor cell death throughout carcinosarcoma cultures within minutes, demonstrating the dramatic sensitivity of these biologically aggressive tumors to CPE. These results were in strong contrast with the lack of sensitivity of normal endometrial 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 uterine carcinosarcoma cells.

In vivo, multiple injections of well-tolerated doses of CPE administered intratumorally led to massive tumor necrosis and dramatically inhibited s.c. tumor growth in SARC-1 SCID mouse xenografts. Importantly, these results were obtained by challenging mice harboring extremely large tumor xenografts of SARC-1 (i.e., 12-week established tumors). This route of administration was chosen because claudin-3 and/or claudin-4 may be expressed in some normal human tissues, including the gut, the lungs, and the kidney (13). Thus, the potential high toxicity of CPE at doses necessary for systemic cancer therapy in animal models may ultimately limit its use in humans to regional applications. Consistent with this view, these results combined with our previous work in ovarian cancer (3) suggest...
that the local/regional administration of well-tolerated doses of CPE may have great potential as a novel treatment modality in patients harboring surgically unresectable chemotherapy-resistant carcinosarcoma. In this regard, further strategies to limit CPE toxicity to normal tissues may include local delivery of the blocking CPE\textsubscript{290-313} peptide fragment to gut and lung via enteral and inhalation routes.

Taken together, our results suggest that CPE-mediated therapy may thus represent a novel, potentially highly effective strategy for the treatment of carcinosarcoma refractory to standard treatment modalities. The future design and implementation of phase I clinical trials in patients harboring chemotherapy-resistant carcinosarcoma will determine the feasibility and validity of this novel therapeutic approach.

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Overexpression of *Clostridium perfringens* Enterotoxin Receptors Claudin-3 and Claudin-4 in Uterine Carcinosarcomas

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