Recombinant CPE fused to tumor necrosis factor targets human ovarian cancer cells expressing the claudin-3 and claudin-4 receptors

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Abstract

Using gene expression profiling, others and we have recently found that claudin-3 (*CLDN3*) and claudin-4 (*CLDN4*) are two of the most highly and consistently upregulated genes in ovarian carcinomas. Because these tight junction proteins are the naturally occurring receptors for *Clostridium perfringens* enterotoxin (CPE), in this study, we used the COOH-terminal 30 amino acids of the CPE ($CPE_{290-319}$), a fragment that is known to retain full binding affinity but have no cytolytic effect, to target tumor necrosis factor (TNF) to ovarian cancers. We constructed a pET32-based vector that expressed the fusion protein, designated here as $CPE_{290-319}$-TNF, in which $CPE_{290-319}$ was fused to TNF at its NH_2-terminal end. Western blotting confirmed presence of both $CPE_{290-319}$ and TNF in the fusion protein. The TNF component in $CPE_{290-319}$-TNF was 5-fold less potent than free TNF as determined by a standard L-929 TNF bioassay. However, the $CPE_{290-319}$-TNF was >6.7-fold more cytotoxic than free TNF to 2008 human ovarian cancer cells, which express both CLDN3 and CLDN4 receptors. shRNAi-mediated knockdown of either *CLDN3* or *CLDN4* expression in 2008 markedly attenuated the cytotoxic effects of $CPE_{290-319}$-TNF. The fusion construct was efficiently delivered into target cells and located in both cytosol and vesicular compartments as assessed by immunofluorescent staining. We conclude that $CPE_{290-319}$ effectively targeted TNF to ovarian cancer cells and is an attractive targeting moiety for development of CPE-based toxins for therapy of ovarian carcinomas that overexpress CLDN3 and CLDN4. [Mol Cancer Ther 2009;8(7):1906–15]

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Introduction

Claudins are the major integral membrane proteins forming the backbone of tight junctions between epithelial cells. The claudin family consists of 23 transmembrane proteins that exhibit distinct tissue- and development-specific expression (1). These proteins can form homodimers or heterodimers to produce paired strands connecting adjacent cells, thereby determining the characteristic permeability properties of different epithelial tissues (2). Using gene expression profiling, others and we have found that claudin-3 (*CLDN3*) and claudin-4 (*CLDN4*) genes are highly expressed in ovarian cancers (3–5). In addition, several other studies have reported aberrant claudin expression in various cancers. Some examples include increased expression of *CLDN3* and *CLDN4* in prostate and uterine cancers (6, 7), and high *CLDN4* expression in pancreatic cancer (8, 9). These two genes are not normally highly expressed in nonmalignant human tissues including the normal ovarian epithelium (10), clearly associating abundance of these two proteins with malignancy. However, the functional importance of such overexpression for the development or progression of ovarian cancer remains uncertain.

Although the exact role that *CLDN3* and *CLDN4* overexpression plays in ovarian cancer is still unclear, these tight junction proteins have recently been shown to be the naturally occurring receptors for *Clostridium perfringens* enterotoxin (CPE), a single polypeptide with a molecular mass of 35 kDa that causes the symptoms associated with *Clostridium perfringens* food poisoning (11). Among >20 members of claudin family, CLDN3 and CLDN4 are the only transmembrane tissue-specific claudin proteins capable of mediating CPE binding and cytolysis (12). Analysis of the CPE structure-function relationship through characterization of the functional properties of enterotoxin fragments has revealed that the COOH-terminal 30 amino acid fragment of CPE ($CPE_{290-319}$) does not cause cytolysis but retains high affinity binding to the claudins; this fragment also completely blocks specific binding of the full-length toxin, thus abolishing cytolysis of susceptible target cells (13). Furthermore, although it remains potentially immunogenic, the C-$CPE_{290-319}$ domain contains fewer antigenic determinants than the COOH-terminal half of the CPE molecule (14). These findings suggest that the CPE peptide fragment might serve to target CPE-based toxins to claudins

on various cancer cells that express high levels of CLDN3 and CLDN4.

Since the discovery of tumor necrosis factor (TNF) α (TNF-α), a 17-kDa protein derived from macrophages, researchers have explored many approaches to harness the potency of TNF and TNF superfamily members to treat human cancers. However, the use of the TNF in cancer therapy is restricted by severe toxicity and limited clinical efficacy against many different tumor types (15). Because of the toxicity associated with delivering TNF systemically at clinically effective doses, strategies are being sought to concentrate the toxin in the tumor. Antibody-mediated delivery of TNF to tumor cells has proven to be a feasible approach (16, 17); however, the relatively large molecular mass of antibody molecules results in inadequate uptake and poor distribution in tumors (18, 19). We have developed a fusion gene encoding a protein containing the COOH-terminal 30 amino acid fragment of CPE and full-length TNF. The $CPE_{290-319}$-TNF fusion gene was cloned into the pET-32a (+) vector and expressed in bacterial host cells. We then characterized the *in vitro* behavior of this fusion protein and evaluated its biological activity compared with that of native TNF. We report here that $CPE_{290-319}$-TNF is more toxic to cells expressing claudin receptors, suggesting that $CPE_{290-319}$ is an effective targeting moiety for delivering TNF to CLDN overexpressing malignant carcinomas.

Materials and Methods

Reagents

Tissue culture media were purchased from Life Technologies, pET bacterial expression systems and recombinant enterokinase (rEK) from Novagen, and metal-affinity resin Ni-NTA agarose from Qiagen. Mouse anti-human TNF monoclonal antibody (anti-huTNF) was purchased from Sigma, and goat polyclonal anti-CPE antibody was obtained from GenWay Biotech, Inc. Mouse anti–claudin-3 and goat anti–claudin-4 antibodies were purchased from Zymed Laboratories, Inc. Antibody to early endosomal antigen 1 (EEA1) was obtained from Santa Cruz Biotechnology. FITC-conjugated and Texas red–labeled secondary antibodies against mouse, rabbit, and goat immunoglobulin were obtained from Jackson ImmunoResearch.

Cells and Cell Culture

The human ovarian carcinoma cell lines 2008 and OVCAR-3 were grown in RPMI 1640 supplemented with 10% fetal bovine serum. The mouse fibroblast cell line L929 (American Type Culture Collection; CCL-1 NCTC clone 929) was maintained in RPMI 1640 (Life Technologies) supplemented with 5% FCS (Sigma-Aldrich), 5×10^{-5} mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 1 mmol/L nonessential amino acids, 2.2 mol/L sodium pyruvate, 10 mmol/L N-2-hydroxyethylpiperazine, N-2-ethanesulfonic acid, and 2.0 g/L sodium bicarbonate (all from Invitrogen). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Construction of the $CPE_{290-319}$-TNF Fusion Gene

$CPE_{290-319}$-TNF was constructed in two-step PCR reaction. The fragment $CPE_{300-319}$ was first fused into the recombinant human TNF cDNA using the plasmid pET-32-TNF as a template with forward primer (5′-ATGAAAGCTAATTCATCATATAGTGGAAATTACCCTTATTCAA TATTATTTCAAAAATTTGGTCTAGAAGTTCG-3′) and reverse primer (5′-CGA*CTCGAG* CTATTAGAGAGCGATGATACC-3′; XhoI site is italicized). The resulting PCR fragment $CPE_{300-319}$-TNF was further extended at its 5′ end by the addition of $CPE_{290-209}$ in a second PCR reaction using the primers (5′-CTACGA*GGTACC*GACGACGACGACAAGAGCTTAGATGC TGGACAATATGTTCTTGTAATGAAAGCTAATTCATC-3′; KpnI site is italicized; 5′-CGA *CTCGAG*CTATTAGAGAGCGATGATACC-3′; XhoI site is italicized). The resulting PCR product containing $CPE_{290-319}$-TNF was digested with KpnI and XhoI and cloned into KpnI/XhoI-digested pET-32a (+) to generate the expression vector pET32-$CPE_{290-319}$-TNF. The constructed plasmid was sequence verified and transformed into *Escherichia coli* strain BL21(DE3) pLysS that were grown to A_{600} = 0.6 at 37°C and induced by addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.4 mmol/L at 30°C for 4 h.

Purification of $CPE_{290-319}$-TNF Fusion Protein

The bacteria were resuspended in lysis buffer [50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, and 10 mmol/L imidazole (pH 8.0)] containing 100 μg/mL lysozyme and sonicated. The lysate was centrifuged at 14,000 *g* for 30 min at 4°C to pellet the cellular debris. The supernatant that contained the soluble fraction of the recombinant protein was loaded onto a Ni-NTA column preequilibrated with the same lysis buffer. After washing the column twice with a wash buffer [50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, and 20 mmol/L imidazole (pH 8.0)], the bound proteins were eluted with a elution buffer containing 50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, and 250 mmol/L imidazole (pH 8.0). Absorbance (280 nm) and SDS-PAGE analyses were done to determine which fraction(s) contained the majority of polyhistidine-tagged (6xHis tag) protein. Fractions were combined and dialyzed against 20 mmol/L Tris-HCl (pH 7.4) and 50 mmol/L NaCl. The fusion protein was incubated with rEK to remove the 6xHis tag. rEK was removed using EKapture Agarose according to manufacturer's instructions. The mixture without rEK was further applied to a Ni-NTA column to remove incompletely digested material, the cleaved NH_2-terminal part of the protein containing the 6xHis tag as well as other contaminating proteins. The final protein product was collected in the flow-through and buffer-exchanged with PBS using a PD-10 column.

L929 TNF Bioassay

The biological activity of TNF was determined by measuring inhibition of the growth of L929 murine fibroblast cells. Log phase cells in a volume of 100 μL were plated in a 96-well tissue culture plates (Falcon) at a density of 5,000 cells per well and incubated overnight at 37°C in a 5% CO_2 atmosphere. Then, 100 μL of serial dilutions of TNF or $CPE_{290-319}$-TNF in PBS were added in the presence

of actinomycin D (0.5 g/mL, Sigma) for 96 h after which 10 μL of Cell Counting kit-8 (Dojindo Molecular Technologies, Inc.) solution was added to each well, and the plates were further incubated for 4 h at 37°C. Plates were read on a microplate ELISA reader at 450 nm. The relative TNF activity retained in $CPE_{290-319}$-TNF was calculated by the ratio of IC_{50} of $CPE_{290-319}$-TNF to IC_{50} of TNF.

Glutaraldehyde Cross-Linking

TNF and $CPE_{290-319}$-TNF equilibrated for 30 min in 20 mmol/L phosphate buffer (pH 7.8). Gluteraldehyde was added to a final concentration of 0.02%, and after 15 min at room temperature, the cross-linking reaction was stopped by the addition of SDS-PAGE sample buffer containing 100 mmol/L glycine. The products of the cross-linking reaction were analyzed by 4% to 15% SDS-PAGE and subsequently characterized by Western blotting using anti-huTNF antibody.

Knockdown of *CLDN3* and *CLDN4* Expression in 2008 Cells Using shRNAi Lentivirus

MISSION Lentiviral Transduction Particles for the *CLDN3* and *CLDN4* knockdown experiments were purchased from Sigma.[3] Briefly, a culture containing 70% to 80% confluent 2008 cells was transduced with shRNAi lentiviral constructs at various multiplicities of infection (MOI) for 24 h. The media containing lentiviral particles was then replaced with fresh media, and after a subsequent 24 h, successfully transduced cells were selected by exposure to 10 μg/mL puromycin. The puromycin-containing media was refreshed every 3 to 4 d until resistant colonies were identified. A minimum of five puromycin-resistant clones was picked and expanded into individual cell lines and assayed for knockdown of CLDN3 and CLDN4 by a quantitative reverse transcription-PCR as described below and Western blot analysis.

Quantification of *CLDN3* or *CLDN4* mRNA by Real-time PCR

Total RNA was extracted with TRIzol reagent (Invitrogen). First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and random primers. Real-time PCR was done using the Bio-Rad iCycler iQ detection system in the presence of SYBR Green I dye (Bio-Rad Laboratories). For the *CLDN3* gene expression, the forward (5′-AAGGTGTACGACTCGCTGCT-3′) and reverse (5′-GTCCTGCACGCAGTTGGT-3′) primers were used for amplification with a iCycler protocol consisting of a denaturation program (95°C for 3 min), amplification and quantification program repeated 40 times (95°C for 10 s and 55°C for 45 s), and melting curve analysis. For the *CLDN4* gene expression, PCR was done with the primers (forward, 5′-AGATGGGTGCCTCGCTCTAC-3′; and reverse, 5′-GGGCCGCCTGCAGGTCCTGC-3′), under the same amplification condition as *CLDN3*. The data were analyzed by using the comparative Ct method, where Ct is the cycle number at which fluorescence first exceeds the threshold. The ΔCt values from each cell line were obtained by subtracting the values for β-actin Ct from the sample Ct. A 1-unit difference of Ct value represents a 2-fold difference in the level of mRNA.

***In vitro* Cytotoxicity of TNF and $CPE_{290-319}$-TNF Against Human Ovarian Carcinoma 2008 Cells and Its *CLDN3* or *CLDN4* Knockdown Sublines**

The cells were plated into 96-well plates at a density of 2,000 cells per well and allowed to adhere overnight. The cells were then exposed to different concentrations of either TNF or purified $CPE_{290-319}$-TNF. After 96 h, the effects of TNF and $CPE_{290-319}$-TNF on the growth of tumor cells in culture were determined by the Cell Counting kit-8 method as described above.

Internalization and Intracellular Distribution of TNF and $CPE_{290-319}$-TNF

Cells were plated onto polylysine-coated 24-well chamber slides at 10^5 cells per well and incubated at 37°C overnight under 5% CO_2 and air. Cells were treated with 1 μmol/L TNF or $CPE_{290-319}$-TNF at 37°C for 6 h, washed with glycine buffer [500 mmol/L NaCl, 0.1 M glycine (pH 2.5)] for 10 min to remove excess fusion protein, neutralized with 0.5 mol/L Tris (pH 7.4) for 5 min, fixed in 3.7% formaldehyde, permeabilized in PBS containing 0.2% Triton X-100, and washed again with PBS for thrice. After nonspecific binding was blocked with 1% bovine serum albumin, cells were incubated with primary antibodies at room temperature for 1 h and then washed thrice in PBS containing 0.1% Tween 20 for 10 min followed by staining with FITC or Texas red–coupled secondary antibodies. After three final washes with PBS containing 0.1% Tween 20, cells were incubated with 0.1% Hoechst 333342 for 15 min and mounted in DABCO mounting medium. Microscopy was done at the University of California San Diego Cancer Center Digital Imaging Shared Resource using a Zeiss LSM510 confocal microscope system (Carl Zeiss, Inc.). Images were captured from 0.8-μm sections by a 63× lens and analyzed by SoftWorx software (Applied Precision, Inc).

Results

Expression and Purification of $CPE_{290-319}$-TNF Fusion Protein

A cDNA coding for the fusion protein $CPE_{290-319}$-TNF was cloned into bacterial expression vector pET32a (+). This vector contains a T7 promoter for high-level expression, followed by a 6xHis tag and an enterokinase cleavage site to permit removal of the 6xHis tag after purification. As shown in Fig. 1A, the recombinant protein $CPE_{290-319}$-TNF was effectively expressed as polyhistidine-tagged protein, and after purification by Ni-NTA immobilized metal-affinity chromatography (IMAC), it showed the expected molecular mass of 37 kDa. The 6xHis tag was then removed by digestion with rEK. One liter of the bacterial culture typically yielded ~2.6 mg of the final purified $CPE_{290-319}$-TNF product. As shown in Fig. 1B, the composition of the final purified fusion protein was confirmed by Western blot analysis using both goat anti-CPE IgG or mouse anti-huTNF IgG and its molecular size was identical to the predicted size of 20 kDa.

[3] http://www.sigma-aldrich.com/missionsearch

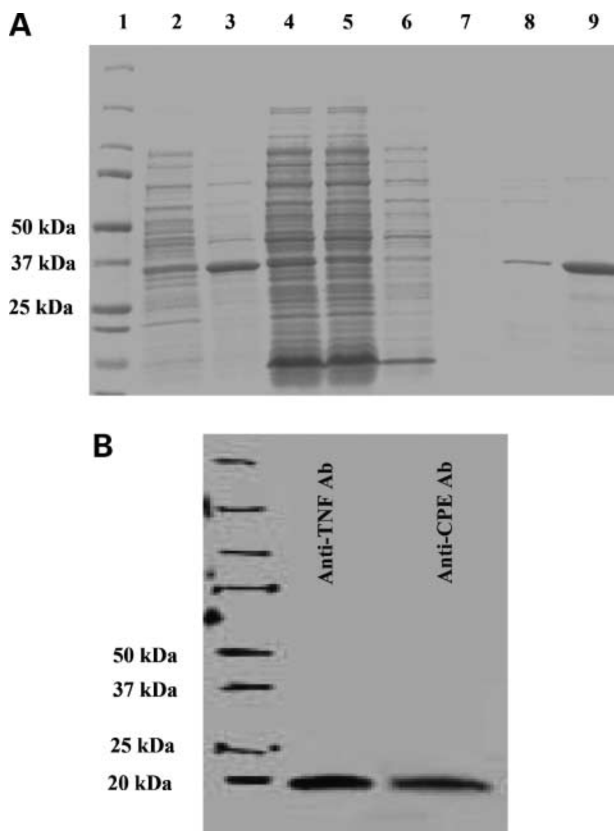

Figure 1. **A,** SDS-PAGE with Coomassie blue staining of $CPE_{290-319}$-TNF under reducing conditions. The gel shows analysis of soluble lysates from noninduced and induced bacteria, and the subsequent steps in purification. The band at 37 kDa in the lysates from induced bacteria represents the 20 kDa $CPE_{290-319}$-TNF construct plus the 17 kDa component containing the 6xHis tag. *Lane 1,* protein markers; *lane 2,* noninduced bacterial whole lysates; *lane 3,* induced bacterial whole lysates; *lane 4,* induced bacterial soluble lysate; *lane 5,* IMAC flow-through; *lane 6 to 8,* 3 sequential IMAC washes; *lane 9,* IMAC eluate. **B,** Western blot analysis of the purified $CPE_{290-319}$-TNF fusion protein. The presence of $CPE_{290-319}$ and TNF component is documented by staining with both anti-CPE and anti-TNF antibodies.

Biological Activity of $CPE_{290-319}$-TNF and Its Oligomeric State

TNF is highly cytotoxic to murine L929 fibroblasts, and this cell line has been routinely used to standardize the biological activity of TNF and TNF-based molecules. The ability of $CPE_{290-319}$-TNF to inhibit the growth of L929 cells was compared with that of native TNF. As shown in Fig. 2A, native TNF had an IC_{50} of 0.6 pmol/L, whereas the $CPE_{290-319}$-TNF was slightly less potent with an IC_{50} of 2.9 pmol/L. Thus, linking TNF to the $CPE_{290-319}$ targeting moiety resulted in a ~5-fold reduction in specific activity compared with that of TNF itself.

This reduction in specific activity could be due to an effect of the $CPE_{290-319}$ component on the ability of TNF to bind to its receptor due to steric hindrance. Alternatively, the $CPE_{290-319}$ fragment may interfere with the trimerization of TNF that occurs in solution and is required for maximal activity. In fact, as shown in Fig. 2B, when native TNF was cross-linked with glutaraldehyde and then examined by gel electrophoresis, it was found to exist entirely as a trimeric complex, whereas under the same conditions, the $CPE_{290-319}$-TNF construct was present primarily as a trimer with small contaminating amounts of monomeric and dimeric material. Thus, at least part of the reduction in the specific activity of the construct compared with free TNF may be due to the presence of incompletely trimerized molecules.

Generation and Characterization of *CLDN3* or *CLDN4* Knockdown Cells

Human 2008 ovarian cancer cells express both CLDN3 and CLDN4 (10). To establish a biological system for testing the potency and selectivity of $CPE_{290-319}$-TNF, the expression of *CLDN3* and *CLDN4* in these cells was constitutively suppressed using a lentivirus expressing an shRNAi targeted to these mRNAs and a puromycin resistance gene. Individual puromycin-resistant clones were expanded and the resulting populations were screened for *CLDN3* or *CLDN4* mRNA expression by reverse transcription-PCR. The clones having the greatest *CLDN3* or *CLDN4* knockdown, designated here as 2008-CLDN3KD-4.5 and 2008-CLDN4-KD-5.5, were used for subsequent experiments. As shown in Fig. 3A, clone 2008-CLDN3KD-4.5 was found to express only 5% as much *CLDN3* mRNA as the parental 2008 cells, and clone 2008-CLDN4KD-5.5 expressed only 14% of *CLDN4* mRNA in the parental 2008 cells (Fig. 3B). The Western

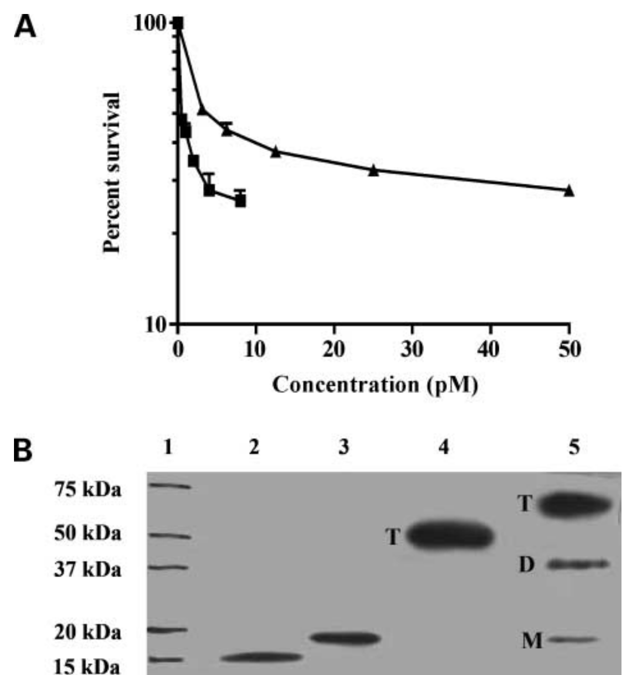

Figure 2. **A,** comparison of the bioactivity of TNF and $CPE_{290-319}$-TNF. L929 cells were treated with increasing concentrations of TNF (■) or $CPE_{290-319}$-TNF (▲) for 96 h. *Points,* the mean of independent experiments, each done with triplicate cultures; *bars,* SE. **B,** SDS-PAGE analysis of TNF and $CPE_{290-319}$-TNF following glutaraldehyde cross-linking. *Lane 1,* molecular mass ladder; *lane 2,* TNF without glutaraldehyde; *lane 3,* $CPE_{290-319}$-TNF without glutaraldehyde; *lane 4,* TNF in the presence of glutaraldehyde; *lane 5,* $CPE_{290-319}$-TNF in the presence of glutaraldehyde. *T,* trimer; *D,* dimer; *M,* monomer.

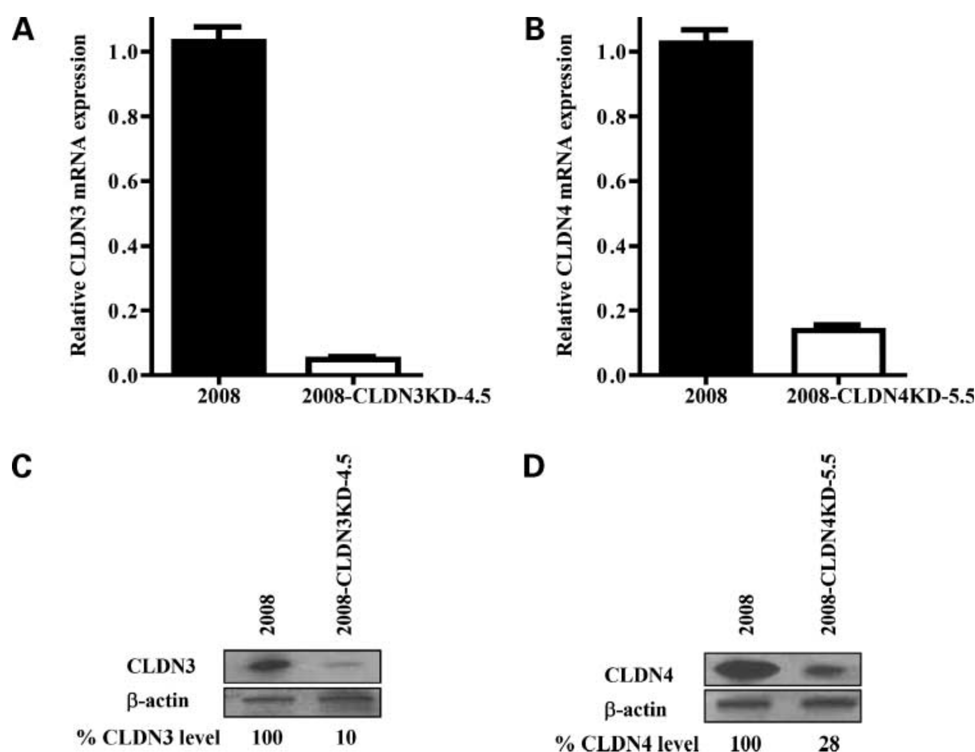

Figure 3. Generation and characterization of clones of 2008 in which *CLDN3* or *CLDN4* expression has been knocked down. **A,** relative *CLDN3* mRNA levels in the parental 2008 cells and the 2008-CLDN3KD-4.5 subline. **B,** relative *CLDN4* mRNA levels in the parental 2008 cells and the 2008-CLDN4KD-5.5 subline. **C,** Western blot analysis of CLDN3 expression in the parental 2008 and 2008-CLDN3KD-4.5 cells. Percent of CLDN3 protein levels in 2008-CLDN3KD-4.5 cells relative to that in the 2008 cells was calculated densitometrically after normalization to the level of β-actin. **D,** Western blot analysis of CLDN4 expression in the parental 2008 and 2008-CLDN4KD5.5 cells. Percent of CLDN4 protein levels in 2008-CLDN4KD-5.5 cells relative to that in the 2008 cells was calculated densitometrically after normalization to the level of β-actin.

blots presented in Fig. 3C and D show that the 95% and 86% reduction in level of the *CLDN3* and *CLDN4* mRNA were accompanied by 90% and 72% reduction, respectively, in the levels of the CLDN3 and CLDN4 proteins as analyzed densitometrically after normalization for β-actin levels. Knockdown of neither CLDN caused a change in the level of expression of TNFR1 as determined by Western blot analysis (Supplementary Fig. S1).[4] Knockdown of both *CLDN3* and *CLDN4* was lethal to these cells and no clones in which both mRNAs were knocked down could be established.

Cytotoxic Effects of $CPE_{290-319}$-TNF

To examine the potency and selectivity of $CPE_{290-319}$-TNF, we compared the cytotoxic effects of the fusion construct and free TNF on the CLDN3- and CLDN4-expressing parental 2008 cells and on the *CLDN3* and *CLDN4* knockdown sublines. The data presented in Fig. 4A show that $CPE_{290-319}$-TNF produced concentration-dependent killing of the parental 2008 tumor cells that express both CLDN3 and CLDN4; the IC_{50} was 1.1 μmol/L. In contrast, native TNF caused only 15% cell death even at the highest concentration tested (4 μmol/L). In comparison, at 4 μmol/L, $CPE_{290-319}$-TNF killed 97% of the 2008 cells. Thus, $CPE_{290-319}$-TNF seemed to be >6.7-fold more cytotoxic than native TNF against the CLDN receptor–positive cells. Similar results were obtained in the OVCAR-3 human ovarian carcinoma (Fig. 4B) that also expresses high levels of both CLDN3 and CLDN4 (10). Knockdown of either *CLDN3* or *CLDN4* expression in the 2008 cells significantly decreased their sensitivity to $CPE_{290-319}$-TNF but had no effect on the response to TNF (Fig. 4C and D). Although $CPE_{290-319}$-TNF still showed slightly more toxicity to *CLDN3* or *CLDN4* knockdown cells compared with TNF, at the highest concentration of 20 μmol/L, $CPE_{290-319}$-TNF only caused 53% and 55% cell death of the *CLDN3* knockdown 2008-CLDN3KD-4.5 and *CLDN4* knockdown 2008-CLDN4KD-5.5 cells, respectively. These results suggest that the cytotoxic activity of the $CPE_{290-319}$-TNF fusion toxin is dependent on the coordinate expression of both CLDN3 and CLDN4 on the tumor cell membrane and that reduction of either is sufficient to impair the relative activity of the construct. To further confirm that enhanced cytotoxicity of $CPE_{290-319}$-TNF is dependent on the CPE moiety, 2008 cells were pretreated with increasing concentrations of free $CPE_{290-319}$ and then exposed to a cytotoxic concentration of $CPE_{290-319}$-TNF. As shown in Supplementary Fig. S2,[4] $CPE_{290-319}$ protected the 2008 cells in a concentration-dependent manner when they were subsequently challenged with 2 μmol/L $CPE_{290-319}$-TNF, whereas preincubation of the cells with bovine serum albumin did not reduce the cytotoxicity of $CPE_{290-319}$-TNF. These results suggest that preincubation with $CPE_{290-319}$ protects 2008 cells by saturating the CLDN receptors that mediate cytotoxicity. To document that $CPE_{290-319}$-TNF triggered apoptosis, 2008 cells were incubated with either 2 μmol/L

[4] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

TNF or $CPE_{290-319}$-TNF for 1 hour and examined for the presence of cleaved and activated caspase-8 by Western blot analysis. As shown in Supplementary Fig. S3,[4] the p26 and p18 forms of cleaved and activated caspase-8 were readily detected following exposure to $CPE_{290-319}$-TNF but not after exposure to TNF.

Internalization and Cellular Distribution of $CPE_{290-319}$-TNF

Immunofluorescent staining followed by confocal imaging was done on 2008 cells treated with 1 μmol/L $CPE_{290-319}$-TNF or TNF for 6 hours and subsequently exposed to an acid buffer to remove any protein nonspecifically associated with the outside of the cell. Internalized toxin was detected using mouse anti-huTNF antibody or goat anti-CPE antibody followed by Texas red–conjugated anti-mouse IgG or FITC-coupled anti-goat IgG. As shown in Fig. 5C and F, cells treated with the $CPE_{290-319}$-TNF fusion protein stained strongly positive with antibodies to either the TNF or the $CPE_{290-319}$ component; however, cells treated with the same concentration of TNF showed no staining (Fig. 5B and E), indicating that the fusion construct is capable of efficient cell binding and rapid internalization into the cells expressing CLDN3 and CLDN4. However, the images in Fig. 5C and F showed both punctuate staining suggestive of localization within vesicular structures and diffuse intracellular staining indicative of cytosolic localization. Analysis of the intracellular vesicular distribution of $CPE_{290-319}$-TNF using costaining with an antibody specific for the early endosomal marker EEA-1 showed extensive colocalization indicating that much of the $CPE_{290-319}$-TNF was trapped in endosomes (Fig. 6). This suggests that the fusion protein seems to enter these cells via a classic receptor-mediated endocytotic process.

Discussion

TNF-α has antitumor activity in a variety of animal models (20); however, clinical trials produced disappointing results. Minimal responses were accompanied by severe hemodynamic side effects and hepatic toxicity (20). There has been speculation that, even when administered at a maximum tolerated dose, the concentration of TNF-α that can be achieved in the tumors is too low to produce meaningful antitumor effects. These results were sufficiently disappointing that studies of systemically delivered single-agent TNF-α were discontinued. TNF-α is now used clinically only via isolated organ perfusions for melanomas and soft tissue sarcomas in combination with conventional antineoplastic agents (21). Thus, if TNF is to be given systemically, it will have to be in a form that is concentrated in the tumor using a delivery system. One approach has been to either conjugate or fuse TNF to antibodies directed at tumor-specific antigens (16, 22–26). Recently, the novel immunocytokine scFv23/TNF targeting Her-2/neu has been shown to sensitize TNF-resistant Her-2/neu–overexpressing breast cancer

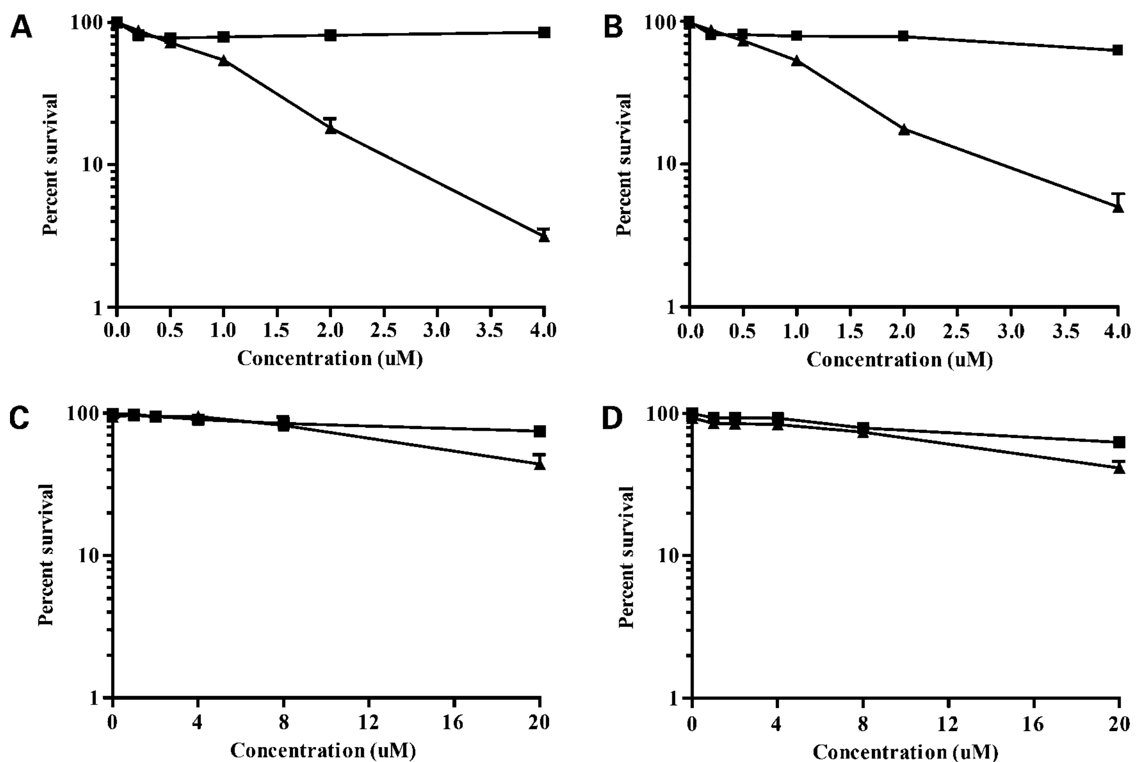

Figure 4. *In vitro* cytotoxicity of $CPE_{290-319}$-TNF and TNF. **A,** 2008 cells; **B,** OVCAR-3 cells; **C,** 2008-CLDN3KD-4.5 cells; and, **D,** and 2008-CLDN4KD-5.5 cells. (■), TNF; (▲), $CPE_{290-319}$-TNF. *Points,* mean of three independent experiments each done with triplicate cultures; *bars,* SE.

cells to TNF-induced apoptosis (27). In addition, an immunocytokine containing TNF and directed at a melanoma-associated tumor antigen has shown impressive *in vitro* cytotoxic effects compared with TNF and produced regression of established melanoma xenografts *in vivo* (16, 17).

Alterations in tight junction structure and function are common in epithelial malignancies (28, 29). Using gene expression profiling, others and we have identified *CLDN3* and *CLDN4* as being among the genes most highly up-regulated in ovarian carcinoma (3–5). A study using an ovarian tissue array has confirmed that CLDN3 and CLDN4 are also highly overexpressed at the protein level in the majority of primary ovarian tumors (10). Overexpression of CLDN3 or CLDN4 in human ovarian surface epithelial cells was reported to increase motility and invasiveness, and small interfering RNA knockdown of either *CLDN3* or *CLDN4* in ovarian cancer cell lines inhibited invasiveness (30). These two tight junction proteins have been described as the low- and high-affinity receptors, respectively, for the cytotoxic CPE (11, 31). Indeed, administration of CPE has been shown to reduce the growth of human ovarian cancers that express high levels of CLDN3 and CLDN4 (32). Ebihara et al. (33) first reported that a COOH-terminal fragment composed of CPE amino acids 184 to 319 could target *Pseudomonas* exotoxin, PSIF, a form of the toxin that lacks the cell binding domain, to CLDN4-expressing tumor cells. Because the receptor binding region of CPE has been reported to lie within just the COOH-terminal 30 amino acids (amino acids 290–319) of CPE (13), abbreviated here as $CPE_{290\text{-}319}$, in this study, we sought to determine whether this fragment alone could target a toxin to CLDN3 or CLDN4.

The cytotoxicity of $CPE_{290\text{-}319}$-TNF against the CLDN3/4-positive human ovarian carcinoma 2008 cells was substantially greater than that of TNF, indicating that specific

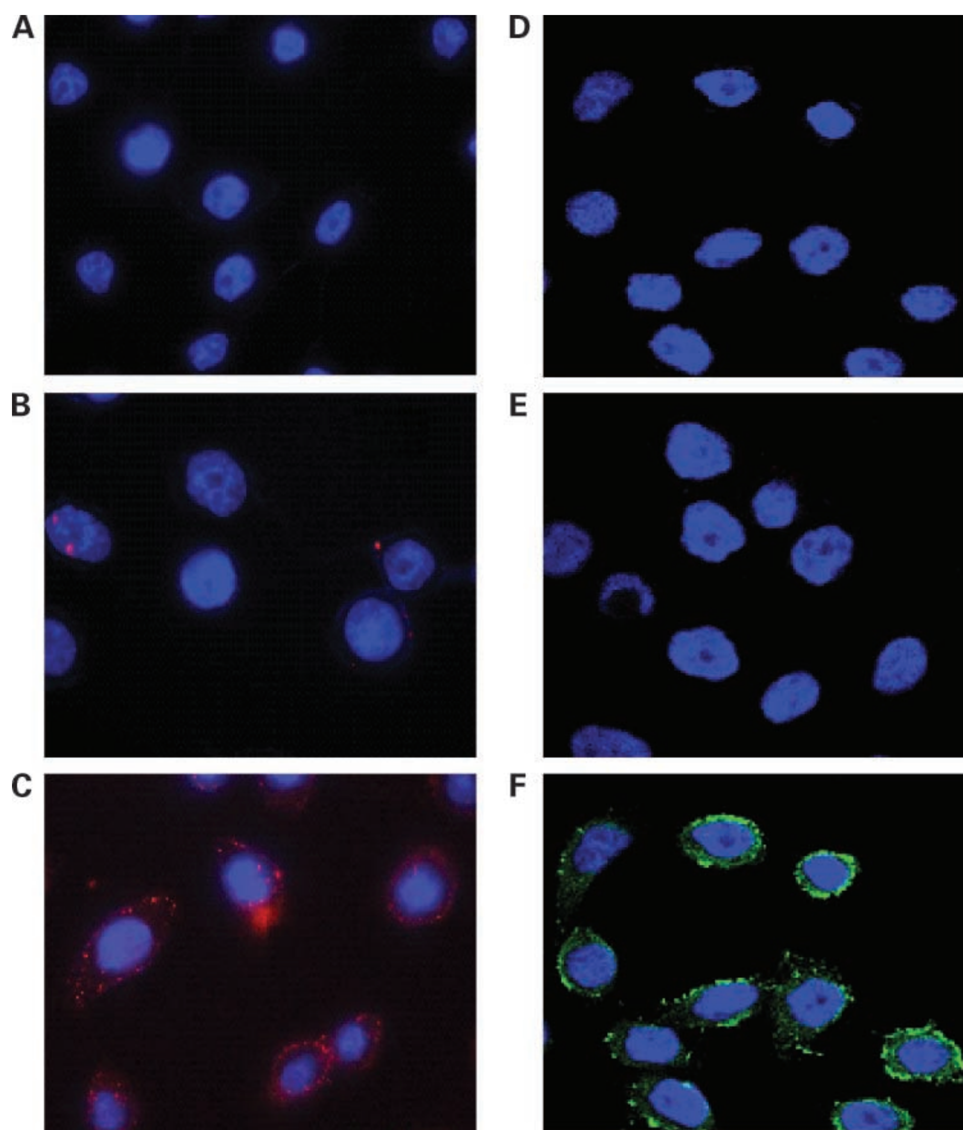

Figure 5. Internalization of $CPE_{290\text{-}319}$-TNF and TNF into 2008 cells. **A** and **D,** no treatment control. **B,** pretreatment with 1 μmol/L TNF, stained with mouse anti-huTNF and Texas red–conjugated anti-mouse IgG. **C,** pretreatment with 1 μmol/L $CPE_{290\text{-}319}$-TNF and stained with mouse anti-huTNF and Texas red–conjugated anti-mouse IgG. **E,** pretreatment with 1 μmol/L TNF and stained with rabbit anti-CPE and FITC-coupled anti-rabbit IgG. **F,** pretreatment with 1 μmol/L $CPE_{290\text{-}319}$-TNF and stained with rabbit anti-CPE and FITC-coupled anti-rabbit IgG. Nuclei are stained with Hoechst 33342 (*blue*).

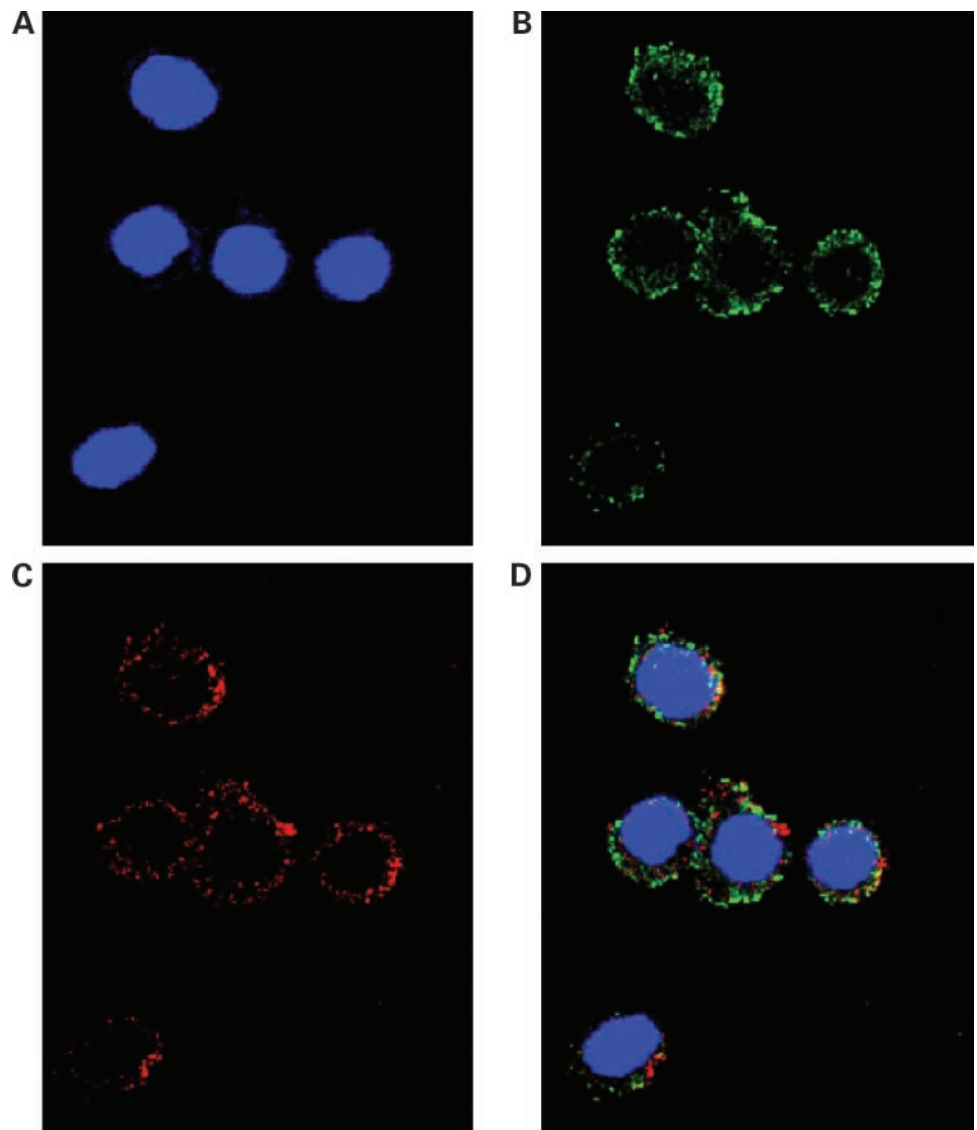

Figure 6. Intracellular distribution of $CPE_{290-319}$-TNF in human 2008 ovarian cancer cells treated with 1 μmol/L $CPE_{290-319}$-TNF for 6 h. **A,** no treatment control. **B,** stained with goat anti-CPE and FITC-conjugated antigoat IgG. **C,** stained with mouse anti-early endosome antigen 1 (EEA1) and Texas red–coupled antimouse secondary antibody. **D,** merged image showing colocalization of $CPE_{290-319}$-TNF with EEA1-positive early endosomal vesicles (*yellow*). Nuclei were stained with Hoechst 33342 (*blue*).

targeting to tumor cells expressing high levels of CLDN3/4 was achieved. Consistent with this finding, shRNAi-mediated knockdown of either *CLDN3* or *CLDN4* expression in 2008 cells resulted in a marked decrease in their sensitivity to $CPE_{290-319}$-TNF but not to TNF. We attempted to knockdown both *CLDN3* and *CLDN4* in 2008 cells but failed to establish such a double-knockdown subline. It is possible that some expression of either of these claudins is essential and that loss of both prevents cell proliferation in culture. This is consistent with studies in transgenic mice where knockdown of both *CLDN1* and *CLDN6* was lethal due to a defect in epithelial barrier function (29, 34).

The biological activity of TNF is mediated mainly by two distinct cell surface TNFR of molecular weight 55 (TNFR1) and 75 (TNFR2) kDa, respectively. TNFR1 was detected by immunostaining in 12 of 12 primary ovarian carcinomas (35), and seems to be primarily responsible for TNF toxicity to ovarian cancer cells (36, 37). We speculate that the inhibitory growth effects of $CPE_{290-319}$-TNF in 2008 cells is mediated by TNFR1 and that the improved activity is due to enhanced valency of TNF induced by the additional binding of the $CPE_{290-319}$ targeting moiety to claudin receptors expressed on the surface of 2008 cells. Deconvoluting microscopy indicated that $CPE_{290-319}$-TNF efficiently delivered TNF into the interior of the target cells, as documented by detection with both anti-huTNF and anti-CPE antibodies, raising the possibility that the intracellular TNF interacts with intracellular TNF receptors to trigger unique downstream signaling pathways resulting in apoptosis. In fact, exposure of 2008 cells to $CPE_{290-319}$-TNF resulted in cleavage and active forms of caspase-8, an effect not produced by an equimolar concentration of TNF.

The soluble active form of TNF is a compact trimer that binds to and clusters high-affinity receptors. The formation of the trimeric form of TNF is critical for the interaction of TNF with TNFR1 and the generation of intracytoplasmic signaling events (38, 39). The $CPE_{290\text{-}319}$-TNF fusion toxin was ~5-fold less cytotoxic to L929 cells than native TNF. That this decrease could be due to inadequate trimerization was showed by chemical cross-linking with glutaraldehyde. In the presence of glutaraldehyde, TNF existed exclusively as trimer. However, following identical treatment with glutaraldehyde, $CPE_{290\text{-}319}$-TNF was found to exist as a mixture of monomer, dimer, and trimer. It is interesting to note that, in spite of ~5-fold loss of TNF biological activity, although not fully trimerized, the $CPE_{290\text{-}319}$-TNF was still >6.7-fold more cytotoxic than native TNF against the CLDN receptor–positive cells, suggesting that further purification of just the trimeric form of $CPE_{290\text{-}319}$-TNF would likely show that it is even more potent. To further document that trimerization is important to the activity of $CPE_{290\text{-}319}$-TNF, we constructed a fusion protein in which the $CPE_{290\text{-}319}$ fragment was attached to the COOH-terminal rather than the NH_2-terminal end of TNF. This TNF-$CPE_{290\text{-}319}$ fusion protein failed to trimerize at all and had no biological activity (data not shown). This finding is in accordance with well-established observation that the COOH-terminal region of TNF is required for trimerization (40).

Because $CPE_{290\text{-}319}$-TNF was more effective at killing claudin-expressing cells, it was expected that $CPE_{290\text{-}319}$-TNF would enter these cells in larger amounts than free TNF. Immunofluorescent staining clearly showed that $CPE_{290\text{-}319}$-TNF was found in much greater abundance than TNF in 2008 cells following a 6-hour exposure to equal concentrations. Analysis of its subcellular distribution showed that much of the intracellular $CPE_{290\text{-}319}$-TNF was located in endosomes where it was positioned to continue to signal cell death via TNFR as shown by prior studies showing that death signaling continues during passage through the endocytic pathway and full activation of the apoptotic signaling cascade by TNF actually requires the endocytosis of TNF-receptor (41, 42). It is noteworthy that $CPE_{290\text{-}319}$-TNF can potentially be internalized either through interaction of the CPE domain with CLDN3/4 receptors or through interaction of the TNF motif with a TNFR. Transmembrane receptors are usually endocytosed via clathrin-coated vesicles dependent on sorting signal sequences present in the receptors. The two most common sorting sequences for clathrin-mediated endocytosis are YXXØ and EXXXLL (where X is any amino acid, and Ø is a bulky hydrophobic residue; ref. 43). Because claudin-4 contains an ALGVLL motif at amino acids 92 to 97 and a YVGW motif at amino acids 165 to 168, a claudin-targeting fusion toxin such as $CPE_{290\text{-}319}$-TNF can likely be internalized via clathrin-mediated endocytosis after binding to CLDN3/4 (33). It has been reported that the endocytosis of claudins occurs during tight-junction remodeling (44). The TNF/receptor complex is also known to be able to internalize via clathrin-coated pits and ends up in secondary lysosymes where it is degraded (40). The fact that $CPE_{290\text{-}319}$-TNF was more toxic to CLDN3/4-expressing cells indicates that the claudin-mediated process is dominant.

In conclusion, we have developed a $CPE_{290\text{-}319}$-TNF fusion toxin that exhibits selective and efficient killing of cells expressing CLDN3/4. This is the first demonstration that the COOH-terminal 30 amino acid domain by itself is sufficient to target a therapeutic toxin to CLDN3- and CLDN4-expressing tumor cells. This study indicates that $CPE_{290\text{-}319}$-TNF has significant potential as a therapeutic agent for the large number of different tumor types that express CLDN3 and/or CLDN4. There is a high probability that the same targeting system can be used to deliver a variety of other toxins leading to a spectrum of new therapeutic agents of high selectivity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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