1. INTRODUCTION

Epithelium plays a pivotal role in protecting the body from various internal and external agents, but sometimes the protective mechanisms of the epithelium can be evaded. For example, most malignant tumors arise from epithelium, and often a very early step in the process of epithelial carcinogenesis is disruption of the cellular polarity of epithelial cells. Consequently, a system that targets depolarized epithelial cells likely will contribute greatly to treating cancer. In addition, the epithelium is the initial physical and immunologic barrier against most pathogens. Using mucosal vaccination to induce immune responses in the mucosal epithelium is a potential key mechanism for preventing infectious diseases. Furthermore, the mucosal epithelium regulates the movement of solutes through intercellular spaces, and strategies to modulate the epithelial barrier for enhancing drug absorption have garnered great interest since the 1960s. In short, epithelial cells are important targets for treating cancer, preventing infectious diseases, and enhancing drug absorption.

Tight junctions (TJs) function as an intercellular seal between epithelial cells. Whereas bicellular TJs contain biochemical complexes including occludin, claudins (CLs), and junctional adhesion molecules, tricellular TJs include at least tricellulin and members of the angulin family. Pharmacologic and pathologic research has revealed that CLs offer considerable potential as targets for the delivery of various therapeutic compounds. For instance, CL-1–knockout mice showed increased epidermal permeability to solutes. In addition, local inhibition of CL-1 in peripheral neurons enhanced drug delivery to those cells. Solutes were able to breach the blood–brain barrier of CL-5–deficient mice. The deregulated expression of CLs in malignant tumors makes these proteins potential targets for antineoplastic agents. Furthermore, administration of Clostridium perfringens enterotoxin (CPE), whose receptor is CL-3 and CL-4, showed antitumor activity. Because CL-4 is highly expressed in the epithelium covering mucosal immune tissues, the delivery of antigen to these tissues using a CL-4 ligand may lead to the development of mucosal vaccines. Therefore CLs are very attractive as targets for drug delivery. However, the hydrophobicity and low immunogenicity of CLs have made it difficult to prepare CL binders, including antibodies, and have delayed the development of CL-targeted drug delivery systems.

Herein, we provide an overview of our efforts to develop CL binders for applications in antitumor therapy and mucosal vaccines.

2. TARGETING CANCER

Most lethal cancers are derived from epithelial tissues. Malignant tumor cells frequently exhibit abnormal TJ functions, followed by deregulation of cellular polarity and intercellular contact—features that are common to both early carcinogenesis and advanced tumors. In addition, CLs are key components of TJs, and some CLs are overexpressed in various types of cancers. For example, CL-4 expression is frequently upregulated in breast, prostate, pancreatic, and ovarian cancers. Although typically nearly inaccessible in well-differentiated normal epithelia, CLs are fairly exposed on malignant tumor cells and therefore may be promising candidates for cancer-targeted therapy.

To investigate whether a CL-targeted strategy offers hope as a cancer-targeting mechanism, we generated a fusion protein that combined C-CPE (the C-terminal fragment of Clostridium perfringens enterotoxin) and a peptide drug (bPTH) across mucosal epithelium. The deregulated expression of CLs in malignant tumors makes these proteins potential targets for antineoplastic agents. Furthermore, administration of Clostridium perfringens enterotoxin (CPE), whose receptor is CL-3 and CL-4, showed antitumor activity. Because CL-4 is highly expressed in the epithelium covering mucosal immune tissues, the delivery of antigen to these tissues using a CL-4 ligand may lead to the development of mucosal vaccines. Therefore CLs are very attractive as targets for drug delivery. However, the hydrophobicity and low immunogenicity of CLs have made it difficult to prepare CL binders, including antibodies, and have delayed the development of CL-targeted drug delivery systems.

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Key words claudin; tight junction; epithelial cell; Clostridium perfringens enterotoxin

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CPE), a CL-4 binder, with protein synthesis inhibitory factor (PSIF). PSIF has been widely used for tumor-targeting therapy but it cannot enter cells on its own. Whereas neither C-CPE nor PSIF alone led to cytotoxicity, our C-CPE–fused PSIF dose-dependently killed CL-4–expressing L cells, reaching >90% cell death at 10 ng/mL; in contrast, C-CPE–fused PSIF showed no cytotoxicity of CL-1, -2, or -5–expressing L cells. To confirm the CL-specificity of C-CPE–PSIF in other cell lines, we tested C-CPE–PSIF in CL-4–positive HepG2 cells and CL-4–negative SK-HEP-1 cells. Whereas C-CPE–PSIF was toxic to HepG2 cells at doses as low as 1 ng/mL, the fusion protein had no effect on SK-HEP-1 cells, even at doses as high as 100 ng/mL. These results indicate that C-CPE–PSIF may be an effective CL-4–targeting cytotoxic agent.

CL-4 is expressed in various tissues, including lung, intestine, liver, and kidney. In normal healthy cells CLs predominantly remain sequestered in TJ complexes, but this localization of CLs is deregulated in some cancers. To investigate whether C-CPE–PSIF can recognize the deregulated localization of CL-4, we next examined the effects of C-CPE–PSIF on cells of the human colon carcinoma line Caco-2, which express CL-4. When confluent, Caco-2 cells form a polarized cell monolayer with well-developed TJs; they are frequently used as a model of polarized cells. We found that although CL-4 protein levels were greater in confluent than subconfluent cultures, C-CPE–PSIF was more toxic (47% cell death at 5 ng/mL) in preconfluent cells, which had fewer TJs. In contrast, cell death in confluent cells with well-developed TJs was not observed at doses as high as 10 ng/mL (Fig. 1). To assess whether cellular polarity affects cell sensitivity to C-CPE–PSIF, we grew Caco-2 monolayers in culture chambers that allowed us to treat the apical and basolateral sides of the cells independently. Addition of C-CPE–PSIF to the basolateral compartment, but not the apical compartment, increased the amount of lactate dehydrogenase (a marker of cytotoxicity) that was released into the medium. Therefore C-CPE–PSIF may specifically recognize cells in which cellular polarity is disrupted.

To evaluate the potential efficacy of a CL-4–targeting strategy, we investigated whether C-CPE–PSIF showed antitumor activity in vivo. We inoculated mice with 4T1 cells, a murine cell line frequently used as a spontaneous lung metastasis model, then treated the mice with C-CPE–PSIF. C-CPE–PSIF significantly suppressed tumor growth (Fig. 2A) and lung metastasis (Fig. 2B) in 4T1-bearing mice. In addition, C-CPE–PSIF did not lead to decreases of body weight and to any apparent biochemical side effects in treated mice. Together, these findings indicate that CL-targeting therapy may
be a potent antitumor strategy.

3. TARGETING THE MUCOSA

Approximately 30 million people worldwide die from infectious diseases each year, thus demonstrating the dire importance of new methods to prevent infectious diseases. Vaccination against infectious diseases is a promising approach because it has few side effects and great preventative and therapeutic effects. Whereas parenteral vaccines injected into patients activate systemic immune responses, they fail to potentiate mucosal immune responses. Moreover, parenteral vaccines show poor patient compliance and poor preventive effects against invasion of pathologic microorganisms through the mucosa. In contrast, administration of mucosal vaccines is noninvasive, thus increasing patient compliance, and these agents potentiate both systemic and mucosal immune responses, thus preventing invasion of pathogens into the body as well as eliminating them and infected cells from the body. Therefore mucosal vaccination is an ideal strategy for protecting against infectious pathogens.2,27)

On the other hand, mucosal administration of antigens

Fig. 3. Involvement of CL-4 in Immune Responses to OVA–C-CPE24)

(A) Schematic illustration of OVA–C–CPE mutant. The construct in which the 16 C-terminal amino acids were deleted from C-CPE (that is, the C-CPE303 mutant) did not bind to CL-4. (B) Interaction of OVA–C–CPE303 with wild-type BV (WT-BV) or CL-1– or CL-4–displaying BV (CL1-BV, CL4-BV) was evaluated by ELISA. (C–F) Induction of immune responses by OVA–C–CPE or OVA–C–CPE303. Mice were immunized intranasally with OVA, OVA–C–CPE, or OVA–C–CPE303 (all doses equivalent to 5 µg OVA) once weekly for 3 weeks. Seven days after the last immunization, the levels of (C) serum IgG, (D) nasal IgA, (E) vaginal IgA, and (F) fecal IgA were measured by ELISA. Data are shown as mean ± S.E.M. (n=4).
alone does not potentiate immune responses, and efficient delivery of antigens to mucosal immune tissues is essential for mucosal vaccination.\textsuperscript{27–29} Mucosa-associated lymphoid tissues (MALTs) in the mucosal epithelium function as the first line of defense against pathogenic invasion \textit{via} the epithelium by activating mucosal immune responses.\textsuperscript{30,31} MALTs contain lymphocytes, M cells, T cells, B cells, and antigen-presenting cells (APCs) and are covered by follicle-associated epithelium (FAE). A specialized M-type epithelial cell in the FAE takes up antigen and presents it to the APCs, which are localized below the M cells, for processing.\textsuperscript{32} Therefore ligands that target FAE or M cells may be useful for mucosal vaccination. In this regard, both FAE and M cells show considerable expression of CL-4,\textsuperscript{19,33} suggesting the potential utility of CL-4–targeting in mucosal vaccination.

To evaluate whether a CL-4–targeting strategy would be effective for mucosal vaccination, we genetically fused C-CPE with ovalbumin (OVA), a popular model antigen for vaccination, to generate OVA–C-CPE. We also prepared OVA–C-CPE303, in which the CL-4–binding region was deleted (Fig. 3A). Like C-CPE, OVA–C-CPE bound to CL-4 but not CL-1, whereas OVA–C-CPE303 did not bind either CL (Fig. 3B). We then intranasally inoculated mice with OVA, OVA–C-CPE, or OVA–C-CPE303 once weekly for 3 weeks and measured OVA-specific serum immunoglobulin G (IgG), nasal IgA, vaginal IgA, and fecal IgA levels at 1 week after the last inoculation. All of these immune responses were activated in mice immunized with OVA–C-CPE but were attenuated in mice that received OVA–C-CPE303 (Figs. 3C–F). These findings suggest that CL-4–targeting is an effective antigen delivery system for mucosal vaccination.\textsuperscript{34}

Antigen-specific immune responses are classified as Th1- and Th2-type responses.\textsuperscript{35,36} To clarify the immune response profiles activated by CL-4–targeting, we investigated serum titers of IgG1 (a Th2 response) and IgG2a (a Th1 response) in mice nasally immunized with OVA–C-CPE or OVA–C-CPE303 and the production of interferon-\(\gamma\) (Th1-specific cytokine) and IL-13 (Th2-specific cytokine) from splenocytes isolated from those mice. Unlike OVA–C-CPE303, OVA–C-CPE markedly increased serum IgG1 and IgG2a titers and the production of interferon-\(\gamma\) and IL-13.\textsuperscript{34} Therefore a CL-4–targeting vaccine may activate both Th1 and Th2 immune responses. To evaluate the immune responses induced by nasal vaccination with OVA–C-CPE, we investigated anti-tumor activity in mice bearing OVA-expressing thymoma cells. Mice were immunized with vehicle, OVA, a mixture of OVA and C-CPE, OVA–C-CPE, or OVA–C-CPE303 once weekly for 3 weeks. Seven days after the last immunization, the mice were inoculated subcutaneously in the right back with EG7 cells (\(1\times10^6\)). Tumor volumes were calculated. Data are given as mean\(\pm\)S.D. (n=4). * Value significantly (\(p<0.05\)) different from that of the vehicle-immunized group.

4. DEVELOPING CL BINDERS

Recent progress in understanding the biochemical structure of TJs has provided new insights into CL-targeted drug development. In addition to their use as potent targets for cancer treatment and mucosal vaccination, CLs likely can be manipulated through the use of C-CPE to facilitate mucosal absorp-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Antitumor Activity Induced by Immunization with OVA–C-CPE in an EG7 Cancer Model, OVA-Expressing Thymoma\textsuperscript{34}}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Selection of C-CPE–Displaying Phage Using CL-4–BV System\textsuperscript{35}}
\end{figure}

A mixture of scFv-phage and C-CPE-phage (ratio, 2:10) was incubated in CL-4–BV-coated immunotubes and the bound phages recovered. Phage clones were identified by PCR amplification followed by agarose gel electrophoresis (sizes of putative PCR products: scFv, 856bp; C-CPE, 523bp). Upper panel, before selection using CL-4–BV-coated immunotubes; lower panel, after selection.
baculovirus (BV) has been developed recently, in which membrane proteins are displayed in their active form. Consequently, we investigated whether CL-displaying BV could be used to screen for CL-binding peptides. In this regard, CL-4-displaying BV interacted with C-CPE but not C-CPE303. We then used CL-4-displaying BV to enrich for C-CPE-phages from a mixture containing scFv-phages and C-CPE-phages (Fig. 5). These findings indicate that CL-displaying BV may be a useful screening system for identifying CL-binding peptides.

Like CL-4, CL-1 is a promising target for modulating mucosal and epidermal barriers, preventing infection by hepatitis C virus, and targeting cancer cells. Accordingly, we used a mutant C-CPE phage library and CL-1-displaying BV to screen for peptides with CL-1-binding activity. After three rounds of screening, we isolated a single promising CL-1-binding candidate, m19, which interacted with both CL-1- and CL-4-displaying BV. Subsequent flow cytometry revealed that m19 binds to cells that express CL-1, CL-2, CL-4, or CL-5 (Fig. 6). Therefore m19 may be the first broadly specific CL

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**Fig. 6.** Flow Cytometric Analysis of Interaction between m19 and CL-Expressing L Cells

CL-expressing L cells were treated with C-CPE or m19; labeled secondary antibodies were used to visualize bound C-CPE or m19 through flow cytometry. Solid histograms indicate the level of reactivity of C-CPE or m19; dotted histograms indicate background binding of the anti-His tag and FITC-labeled antibodies without C-CPE or m19.
Because understanding the mode of action of m19 would yield information useful in the development of CL binders, we performed X-ray structural analysis to determine the structures and electrostatic surface maps of m19 and C-PCE. Whereas m19 and C-PCE were structurally similar, the electrostatic maps of their CL-binding regions were greatly different. The binding region of C-PCE is negatively charged, whereas that of m19 is positively charged. Analysis of a series of chimeras revealed that sensitivity to CPE was determined by the region from Asn149 to Met160 in CLs (that is, the CPE sensitivity-related region; CPE-SR). The isoelectric point (pl) values of CPE-SRs in CL-4, CL-1, CL-2, and CL-5 were revealed as 9.70, 4.18, 4.18, and 4.18, respectively. Taken together, these findings suggest that varying the electrostatic charge of the CL-binding region of C-PCE may be a useful method to create CL binders.

5. PERSPECTIVES OF CL-TARGETED DRUG DELIVERY

Applications of C-PCE and its derivatives are limited because of their origins in enterotoxin. Indeed, repeated administration of C-PCE induced production of antibodies in mice. Therefore development of bioconjugate CLs and ligands such as chemicals, peptides, and antibodies is crucial for their application as therapeutics.

Two groups have developed anti-CL antibodies recently. An antibody directed against the extracellular loop region of CL-4 demonstrated antibody-dependent cellular cytotoxicity and in vivo antitumor activity. Another group created anti-CL-1 antibodies that prevented the infection of human hepatocytes by hepatitis C virus. Although these successes will encourage further development of CL antibodies, the high costs associated with this process likely will limit the clinical application of these reagents to the treatment of widespread diseases such as cancer and hepatitis C. In addition, CL-binding chemicals or peptides must be created to capitalize on the capacity for noninvasive administration and drug delivery to peripheral and central neurons through modulation of CLs. To this end, the three-dimensional structures of CLs need to be determined, and the process of developing antibodies to CLs may yield insights into their solubilization and crystallization. Furthermore, CL-displaying BV may be a valuable tool for screening of candidate CL binders, as mentioned previously.

The development of CL-targeting systems is in its very early stages, and additional applications of CL-targeted drug delivery remain to be proposed. We believe that the current problems regarding the development of CL binders will be overcome and that the promise of the clinical value of CL-targeted pharmaceutical therapies will be realized.

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