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Inhibition of Tumor Metastasis: Functional Immune Modulation of the CUB Domain Containing Protein 1

Keisuke Fukuchi,[†,‡,§] Sebastian C. J. Steiniger,[†,‡,∥] Elena Deryugina,[⊥] Ying Liu,[†] Colin A. Lowery,[†] Christian Gloeckner,[†] Bin Zhou,[†] Gunnar F. Kaufmann,[†] James P. Quigley,[⊥] and Kim D. Janda*[,†,#]

The Skaggs Institute for Chemical Biology, Departments of Chemistry, Immunology and Microbial Science, and Cell Biology, and Worm Institute for Research and Medicine, The Scripps Research Institute, La Jolla, California 92037

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Abstract: Despite significant progress and notable successes in tumor therapy, malignant disease remains an extremely difficult problem in today's health care setting. There is, however, an increasing application of new therapies targeting proteins specifically upregulated on tumor cells. These innovative therapeutic approaches are aimed at molecules that contribute to malignant development and progression but spare normal tissues. The CUB domain containing protein 1 (CDCP1) is such a tumor-associated protein and, thus, a potential candidate for targeted cancer immunotherapy. Herein, we describe the generation of function-blocking human antibodies against CDCP1 that were obtained from human scFv phage display libraries using subtractive panning protocols on CDCP1 expressing cancer cells and immunopurified CDCP1 protein. One of the isolated anti-CDCP1 antibodies, namely, C20Fc, efficiently blocked experimental metastasis of human carcinoma cells, including HeLa cells stably transfected with CDCP1 and prostate carcinoma cells PC-hi/diss naturally expressing CDCP1, in both chick embryo and mouse model systems. The C20Fc antibody also reduced colony formation of CDCP1 expressing cells in a soft agar assay for anchorage-independent cell growth. Specific targeting of CDCP1 by C20Fc mediated the delivery of a toxin-conjugated antibody complex, thus, providing evidence for antibody internalization and specific killing of CDCP1-positive tumor cells. Our findings indicate a functional role for CDCP1 in human cancer and underscore the therapeutic potential of function-blocking anti-CDCP1 antibodies targeting both primary and metastatic carcinoma cells.

Keywords: Immunotherapy; CDCP-1; metastasis; human antibodies; cancer; phage display

Introduction

In 2001, the CUB domain containing protein 1 (CDCP1) was identified as a human tumor-associated gene using representational difference analysis and cDNA chip technology.[1] Subsequently, CDCP1 protein levels were shown to be elevated in metastatic human tumor cell lines and determined to be the target of the murine monoclonal antibody (mAb) 41-2.[2] At present, the functional importance of CDCP1 has been reported in patients with metastatic gastric, lung and renal cell carcinomas as well as in a number

* Corresponding author. Mailing address: The Scripps Research Institute, Department of Chemistry, 10550 North Torrey Pines Rd., BCC-582, La Jolla, CA 92037. Tel: (858) 784-2516. Fax: (858) 784-2595. E-mail: kdjanda@scripps.edu.

† The Skaggs Institute for Chemical Biology and Departments of Chemistry and Immunology and Microbial Science.

‡ These authors contributed equally to this work.

§ Current address: Daiichi Sankyo, 1-2-58, Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan.

∥ Current address: XBiotech, 8201 E Riverside Drive, Austin, TX 78744.

⊥ Department of Cell Biology.

Worm Institute for Research and Medicine.

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of epithelial cancer cell lines.[3−13] Importantly, CDCP1 has also been demonstrated to be expressed on cells phenotypically identical to hematopoietic stem/progenitor cells, mesenchymal stem/progenitor cells (MSCs) and neural progenitor cells (NPCs).[14,15] However, the exact functional role of CDCP1 in cancer and stem cells is still poorly understood.

CDCP1 is a single pass transmembrane protein, containing three CUB domains with the extracellular portion most likely involved in cell−cell or cell−extracellular matrix interactions.[16] It promotes invasion and peritoneal dissemination of cancer cells through the regulation of cell migration and anchorage independence.[6] Notably, CDCP1 has been classified as a Src family kinase-binding phosphoprotein, regulating both cell adhesion and resistance to anoikis of cancer cells.[2−4] However, tyrosine phosphorylation of CDCP1 has also been shown to be adhesion-dependent and regulated by protease cleavage in epithelial carcinomas.[3,17] Furthermore, tyrosine phosphorylation is required for the binding of PKCδ to CDCP1.[18]

Several murine mAbs have been generated against CDCP1 by whole cell immunization, and have exhibited function-blocking activity in the chick embryo and mouse metastasis model systems.[2,7,11] However, human mAbs specifically targeting CDCP1 expressed by human tumor cells have not been reported and, if generated, such antibodies would have clinical relevance.

Materials and Methods

Cell Lines and Culture Conditions. The human cervix adenocarcinoma epithelial cell line HeLa (ATCC CCL 2), human prostate carcinoma epithelial cell line PC-3 (ATCC CRL 1435) and African green monkey kidney fibroblast-like cell line COS-1 (ATCC CRL 1650) were purchased from American Type Culture Collection (ATCC, Manassas, VA). A high disseminating variant of the prostate carcinoma PC-3 cell line (PC-hi/diss) was generated by serial passaging of primary tumors developed in chick embryos as described.[19] Tumor cells were cultured in DMEM supplemented with 10% fetal calf serum (D-10), 2 mM GlutaMax and antibiotics (Invitrogen, Carlsbad, CA).

Antibodies. Murine anti-CDCP1 mAb 41-2 was generated by subtractive immunization as described.[2] Murine antihuman transferrin receptor antibody was purchased from eBioscience (San Diego, CA). Normal mouse IgG was purchased from Jackson ImmunoResearch, Inc. (West Grove,

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PA). The control fully human antibody was an antianthrax spore fusion scFv-Fc antibody A4Fc made in our laboratory. Saporin-conjugated goat antimouse IgG (Mab-ZAP) and goat antihuman IgG (Hum-ZAP) secondary antibodies were purchased from Advanced Targeting Systems (San Diego, CA).

Generation of CDCP1 Expressing HeLa Cells. HeLa cells overexpressing CDCP1 protein (HeLa-CDCP1) were generated from parental carcinoma cells, previously demonstrated not to express any detectable CDCP1.[2] HeLa-CDCP1 cells were generated by stable transfection of CDCP1 cDNA in the pcDNA3.1-neo vector (Invitrogen) using Lipofectamine 2000 as described.[11] Several CDCP1-positive, drug resistant clones were combined to generate a HeLa-CDCP1 cell line. Control HeLa-neo cells were generated by transfection of parental cells with the empty vector. Expression of CDCP1 on the cell surface was confirmed using flow cytometry and Western blot analysis with a mouse anti-CDCP1 mAb, 41-2.[2,11]

Combinational Panning. Phage library panning was conducted as previously described.[20,21] In short, before each round of panning, the library was subtracted with CDCP1-negative HeLa-neo cells. A mixture of human scFv phage libraries (5×10^{12} cfu) was incubated with 5×10^7 HeLa-neo cells in 10 mL of culture medium for 1 h at 4 °C. In panning rounds 1−4 the subtracted scFv phage particles were added to HeLa-CDCP1 cells. The cells were incubated with the subtracted scFv phage library for 1 h at 4 °C, washed 5 times with 10 mL of ice-cold PBS, collected by a cell scraper and resuspended in 10 mL of ice-cold PBS. Alternatively, in the third and fourth rounds of panning, subtracted phages were panned on CDCP1 protein captured on STAR coated tubes (NUNC) for 1 h at 4 °C. For CDCP1 capturing, the tubes were first incubated with mouse anti-CDCP1 mAb 41-2 at a concentration of 100 μg/mL in PBS for 2 h at room temperature, washed with PBS, and blocked with 2% casein-PBS. The cell lysate generated from approximately 3×10^7 HeLa-CDCP1 cells was added to mAb 41-2-coated tubes. Following incubation for 1 h at 4 °C, the tubes were washed 3× with PBS and phage were added for 1 h at 4 °C. Upon washing 3× with PBS, the bound scFv-phage were eluted with 1 mL of elution buffer (100 mM HCl, adjusted to pH 2.2 with glycine) for 10 min at room temperature followed by neutralization with 50 μL of 2 M Tris-HCl, pH 8.9. The eluted phage were incubated with 10 mL of *Escherichia coli* TG1 cells in exponential growth. The infected cells were collected by centrifugation (3000 rpm for 5 min), resuspended in 100 μL of Super broth (SB) broth medium, plated on 2% glucose, 100 μg/mL carbenicillin-containing Luria−Bertani (LB) broth agar plates, and incubated at 37 °C. After overnight incubation, the plates were scraped with 1 mL of SB medium supplemented with 15% glycerol, and 100 μL of the resulting suspension was inoculated into 20 mL of SB medium supplemented with 2% glucose, 20 mM $MgCl_2$, and 100 μg/mL carbenicillin. The culture was incubated at 37 °C, and the phagemid rescued by addition of 100 μL of VCSM13 helper phage (10^{11} pfu/mL; Stratagene), followed by incubation at 37 °C for 30 min without shaking and an additional 30 min incubation at the same temperature with agitation. The cells were then collected by centrifugation (3000 rpm for 15 min), resuspended in 100 mL of SB supplemented with 20 mM $MgCl_2$, 100 μg/mL carbenicillin, and 79 μg/mL kanamycin, and incubated at 28 °C overnight with shaking. The scFv-phage were purified by standard PEG precipitation.[22]

Selection of Positive Antibody-Phage Clones by ELISA. HeLa-CDCP1 cells and HeLa-neo cells were incubated at 5×10^4 cells per 0.1 mL 10% fetal bovine serum (FBS) containing DMEM media in 96-well microtiter plates. After overnight incubation, 25 μL of scFv-phage solution were added to each well containing adherent HeLa cells and incubated for 1 h at 4 °C. The plates were washed, 2×, with PBS containing 5% FBS, followed by incubation with HRP conjugated anti-M13 antibody (1:1000 in 5% FBS containing PBS) for 2 h at 4 °C. Following additional washes, the HRP substrate (TMB, Pierce) was added to the plates, the reaction was stopped by addition of 2 M H_2SO_4 when an adequate signal was reached, and the absorbance was read at 450 nm.

Generation and Purification of scFv-Fc Fusion Antibodies. The scFv gene fragments were subcloned into a pSec-Fc vector (derived from the original pSec vector, Invitrogen) containing the human antibody constant regions C_H2 and C_H3 derived from human IgG1 as well as *Sfi*I restriction sites to allow for subcloning of the scFv gene. The obtained plasmids were purified using EndFree plasmid Maxi Kit (Qiagen). The plasmid was transfected into 1×10^7 293 cells with Fugene 6 (Roche). Approximately 16 h later, medium was exchanged for DMEM supplemented with 10% ultralow IgG serum (Invitrogen) and 4 mM GlutaMax (Invitrogen). Transfected cells were incubated for 1 week at 37 °C in 5% CO_2, conditioned medium harvested and filtered through a 0.45 μm filter, and secreted human Fc-tagged scFv antibody (scFv-Fc fusion antibody) was purified using an HiTrap Mabselect Sure column (GE Healthcare, Piscataway, NJ) following the manufacturer's instructions.

Flow Cytometry. Primary antibodies or purified scFv-Fc fusion antibodies were incubated with HeLa-CDCP1 and HeLa-neo cells (5×10^5 cells in 100 μL) on ice for 30 min. After washing with PBS, the cells were stained with FITC-conjugated goat antihuman IgG or anti-M13 phage antibody. Analysis of antibody binding was performed with a Becton

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Dickinson LSRII flow cytometric analyzer. The geometric mean of fluorescence intensity (geomean) was calculated using FlowJo software (TreeStar, Inc., Ashland, OR).

In Vitro Antibody Internalization Assay. The scFv-Fc antibodies or mouse monoclonal antibodies at 10 μg/mL were preincubated under shaking with the same concentration (1:1) of Hum-ZAP (Advanced Targeting Systems) or Mab-ZAP (Advanced Targeting Systems) in DMEM for 30 min at room temperature. These mixtures were used in the cell proliferation assays. Thus, PC-hi/diss, HeLa-CDCP1 or HeLa-neo cells were plated in white 96-well microplate wells at 2,000 cells/45 μL. Next, a mixture of 400 ng of primary antibody with 400 ng of either the Mab-ZAP or Hum-ZAP secondary antibody with PBS as the vehicle instead of a secondary antibody was added in a volume of 5 μL for a final volume of 50 μL. Ligation and internalization of the primary antibody resulted in delivery of the antibody conjugated saporin to the cell interior followed by cell killing. Plates were incubated for 48 h at 37 °C in the presence of 5% CO_2. Cell viability was assayed using a CellTiter GLO cell proliferation assay according to the manufacturer's instructions (Promega), and the luminescence was determined in a Molecular Devices V_{max} microplate reader. Percent cell viability was calculated by assigning the average of the readings from secondary antibody goat IgG-SAP isotype controls with PBS as 100% cell viability. As a positive antibody control for internalization, the anti-transferrin receptor antibody OKT9 (eBioscience) was utilized.

Anchorage Independence Assay. Approximately 1 mL of complete DMEM containing 0.3% low melting point agarose (LMA) was added per well of a 6-well plate and placed at 4 °C until the medium solidified. Plates were warmed at 37 °C, and then 1 mL of DMEM with 0.3% LMA, 200 μg of antibody and 1×10^4 cells were added to the solidified agarose layers. The plates were incubated for one hour at 4 °C and then transferred to a 37 °C incubator containing 5% CO_2. Every three days 0.5 mL of fresh medium containing 0.3% LMA was added to each well and let solidify for one hour at 4 °C. The cells were allowed to grow for 2 weeks in a cell culture incubator. The cell colonies were counted in 8 different areas of each well within the microscope field of view. The mean and SD were calculated per each condition and compared by Student's t test for $P < 0.05$ to determine statistical significance between data sets.

Experimental Metastasis in Chick Embryos. Experimental metastasis was performed in chick embryos developing in fertilized SPAFAS White Leghorn eggs (Charles River). On day 12 of embryo development, 5×10^4 HeLa-CDCP1 or PC-hi/diss tumor cells were injected into the allantoic vein as described.[11] CDCP1 specific or control human antibodies were inoculated iv at 25 μg per embryo at the time of cell injections. Five days later, on day 17 of embryo development, portions of the CAM were harvested to determine numbers of human tumor cells that colonized the CAM tissue.

Experimental Metastasis in Mice. Female 6−8-week-old immunodeficient NOD-SCID mice (TSRI) were injected iv with 1×10^6 HeLa or 5×10^5 PC-hi/diss cells. At the time of cell injection, 100 μg of human anti-CDCP1 or control antibodies was inoculated per animal. Additional antibody injections were performed ip on days 2 and 3 following cell inoculations. Four weeks (HeLa cells) or 8 weeks (PC-hi/diss) later, the mice were sacrificed and lungs were harvested to determine numbers of human tumor cells by quantitative Alu-PCR.

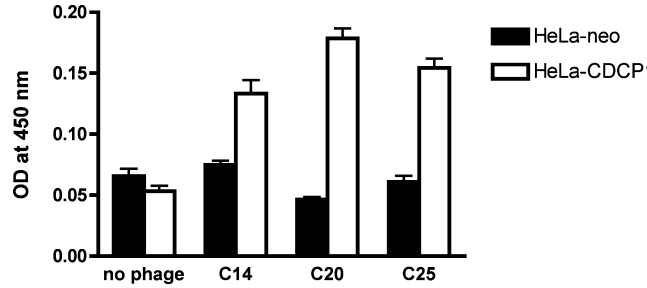

Figure 1. Selected scFv antibodies on phage by whole cell ELISA. Positive scFv antibody expressing phage clones were selected by whole cell ELISA with CDCP1 expressing HeLa cells (HeLa-CDCP1) and Neo expressing HeLa cells (HeLa-neo). Data are the mean of triplicate wells. Error bars indicate the SD.

Quantitation of Human Tumor Cells by Real-Time Quantitative PCR (qPCR). Numbers of human cells within murine or chick embryo tissues were determined by qPCR performed using a standard curve generated by serial dilutions of human tumor cells within a constant number (10^6) of chick embryo or mouse fibroblasts essentially as described.[11] Each assay included negative controls (i.e., water and tissue samples from the embryos that did not receive human cells), and experimental samples in duplicate.

Results

Selection of Human Anti-CDCP1 Single Chain Fv Antibodies. To generate anti-CDCP1 human single chain Fv antibodies (scFv) that recognize different epitopes on the CDCP1 molecule, two alternative panning strategies were utilized. The first approach employed phage panning on HeLa-CDCP1 cells that were stably transfected with a CDCP1 expression vector. After four rounds of panning, one scFv phage-displayed antibody was selected, namely, C25, which demonstrated specific binding to HeLa-CDCP1 as determined by whole cell ELISA (Figure 1). Alternatively, panning was performed on CDCP1 protein immunocaptured by anti-CDCP1 mouse mAb 41-2.[2] This panning approach resulted in the selection of antibodies C14 and C20 that specifically bound to HeLa-CDCP1 cells, but not to HeLa-neo cells (Figure 1).

Sequence analysis for three positive clones demonstrated that each scFv was an independent clone expressing a unique amino acid sequence (Figure 2). Alignment of the amino acid sequences of individual scFv showed that there was no significant sequence identity within the CDR regions of the heavy chain. However, the clones C14 and C25 had identical

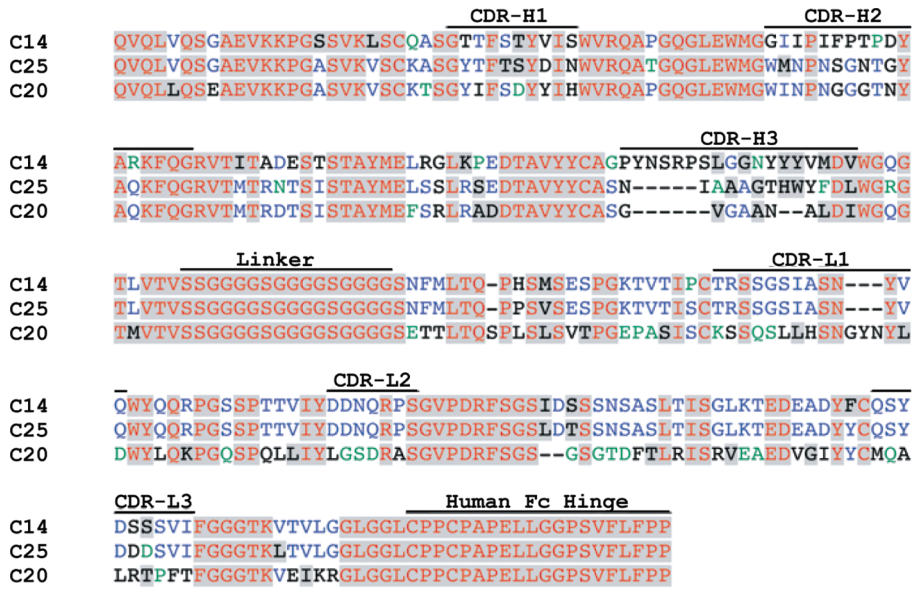

Figure 2. Alignment of anti-CDCP1 antibodies. Multiple sequence alignment was performed with AlignX (Invitrogen). Identical residues of three antibodies are shown in red, of two antibodies are shown in blue. Different residues are shown in black and weakly similar in green. CDR regions of antibodies, linker and the Fc hinge are indicated above the sequences.

CDR L1 and L2 sequences and also exhibited strong similarity within CDR L3.

Generation of Anti-CDCP1 scFv-Fc Antibodies. In order to produce stable antibodies for in vitro and in vivo function blocking analyses, the human IgG Fc domain was appended to scFvs C25, C14 and C20 by subcloning the antibody genes into an expression vector containing the human Fc gene. The C-terminal Fc-fused antibodies (scFv-Fc) were expressed in 293 human kidney cancer cells and purified using a protein G column. The specificity of antibody binding was evaluated by flow cytometry with HeLa-CDCP1 and HeLa-neo cells (Figure 3). Fully human scFv-Fc fusion antibodies, i.e. C14Fc, C20Fc and C25Fc, demonstrated a specific high fluorescence signal with HeLa-CDCP1 cells and no signal with CDCP1-negative HeLa-neo cells.

Evaluation of Anti-CDCP1 Antibodies in an Experimental Metastasis Model in Chick Embryos. To evaluate antimetastasis activity of the anti-CDCP1 antibodies, a human tumor/chick embryo experimental model system was examined (reviewed in ref 11). This assay is based on inoculation of human cancer cells into the allantoic vein of the chick embryo and subsequent analysis of tumor cell colonization of the chorioallantoic membrane (CAM), serving as a lung analogue for the embryo. Thus, 5×10^4 HeLa-CDCP1 transfected cells, or PC-hi/diss cells naturally expressing CDCP1, were injected iv along with 25 μg of control A4Fc or individual anti-CDCP1 scFv-Fc. The embryos were sacrificed on day 5 after initial injections, and the number of human cells colonizing the CAM was determined by Alu-qPCR (Figure 4A). Analysis of tumor cell colonization showed that all three anti-CDCP1 antibodies substantially inhibited both HeLa-CDCP1 (Figure 4A, left panel) and PC-hi/diss (Figure 4B, right panel) cells, with C20Fc demonstrating maximal and significant inhibition down to 15−45% of control scFv-Fc.

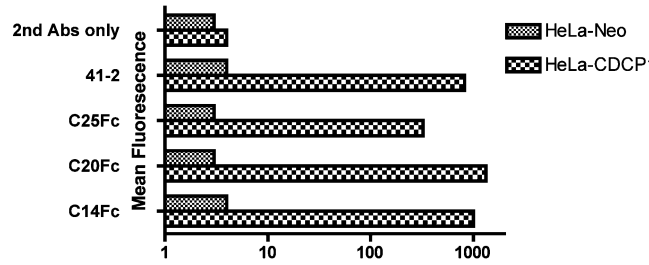

Figure 3. Binding of scFv-Fc antibodies to CDCP1 positive cells. scFv-Fc antibodies recognize cancer cells based on their expression of CDCP1. Several independent experiments on each of these cell types gave similar results. Shown are flow cytometric analyses with binding of scFv-Fc antibodies to cells detected as fluorescence signal of fluorescein labeled antihuman IgG Fc goat 2nd antibody. 41-2 mouse monoclonal antibody was used as a positive control of anti-CDCP1 binding using a fluorescein labeled antimouse IgG goat 2nd antibody.

Evaluation of Anti-CDCP1 scFv-Fc Antibodies in an Experimental Tumor Metastasis Model in Mice. Having demonstrated the efficiency of human anti-CDCP1 by reducing experimental metastasis in chick embryos, we studied the effect on tumor cell colonization in the mouse model. Since C20Fc exhibited maximal inhibitory activity against both carcinoma cell types in the chick embryo (Figure 4A), this antibody was chosen for further evaluation of its function blocking activity in experimental metastasis in mice.

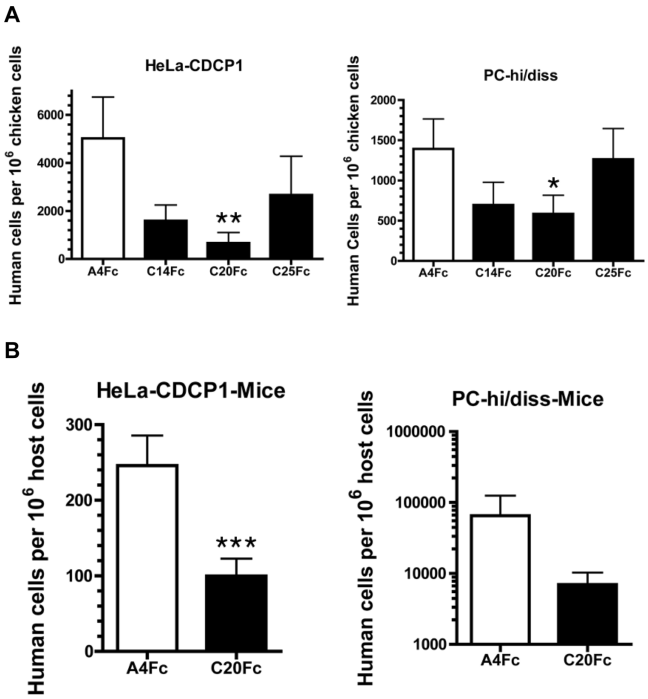

Figure 4. Experimental metastasis of CDCP1 positive cells in chick embryos (A) and mice (B) is modulated by anti-CDCP1 C20Fc. (A) HeLa-CDCP1 cells (left panel) or PC-hi/diss (right panel) were injected iv into chick embryos (5×10^4 cells per embryo) along with 25 μg of control A4Fc or anti-CDCP1 scFv-Fc C14Fc, C20Fc and C25Fc. On day 5 following inoculations, the levels of CAM colonization were determined by Alu-qPCR. *, $P < 0.05$ in unpaired *t* test with Welch's correction; **, $P < 0.05$ in two-tailed unpaired *t* test. (B) HeLa-CDCP1 cells (left panel, 1×10^6 cells/mouse) or PC-hi/diss (right panel, 5×10^5 cells/mouse) were injected iv into immunodeficient NOD-SCID mice (4−6 mice per group) along with 100 μg of control A4Fc or anti-CDCP1 C20Fc. Additional injections of corresponding antibodies were performed ip on days 1 and 2 after cell injections. Levels of lung colonization by tumor cells were determined 4 weeks (HeLa-CDCP1 cells) and 8 weeks (PC-hi/diss cells) after cell inoculations. Data are means ± SEM of numbers of human cells per 10^6 host cells determined by qPCR. ***, $P < 0.05$ in a two-tailed unpaired *t* test.

Treatment with C20Fc caused a nearly complete inhibition (>85%) of metastatic burden in the lungs of mice injected with highly colonizing PC3-hi/diss (Figure 4B, right panel) and approximately 60% inhibition of lung metastasis in mice injected with low colonizing HeLa-CDCP1 cells (Figure 4B, left panel), thereby validating our initial inhibitory effects observed in the chick embryo model.

Anchorage Independent Growth of CDCP1-Expressing Cells Is Suppressed by Anti-CDCP1 Antibody C20Fc. The inhibitory effects of the C20Fc antibody on tumor cell survival and growth were evaluated in an anchorage independence assay where inhibition of colony formation is considered a stringent in vitro test of the anticancer activity of an antitumor drug candidate. Antibody C20Fc reduced

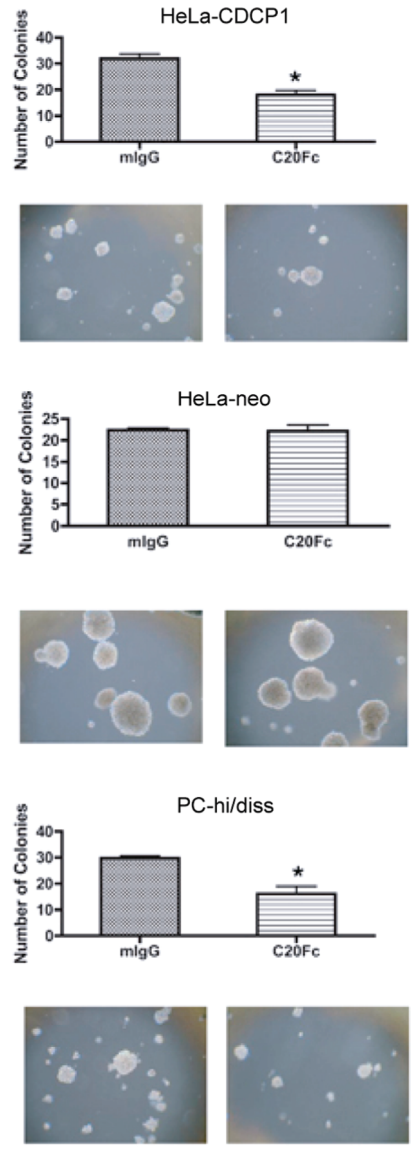

Figure 5. Antibody effect for anchorage independent colony formation of cancer cells in soft agar. Colony formation of CDCP1 expressing cell lines, PC-hi/diss and HeLa-CDCP1 or CDCP1 negative cell line, HeLa-neo, was studied in 0.3% LMA containing complete media for 2 weeks. The number of colonies was counted in eight random fields from each plate using a microscope. Data shown are the mean ± SD for the number of colonies in each field. Significant difference is indicated by asterisks (*) when *P* value was less than 0.05 (*t* test).

significantly (by approximately 50%) colony formation of CDCP1 positive cells, both HeLa-CDCP1 and PC-hi/diss, but did not affect the growth of CDCP1 negative, HeLa-neo cells (Figure 5).

CDCP1-C20Fc-Mediated Cell Cytotoxicity. To further evaluate targeting of CDCP1 by human anti-CDCP1 antibodies in the context of antibody-mediated therapy, we analyzed whether engagement of CDCP1 by C20Fc induced antibody−ligand complex internalization. To this end, a cell-killing assay was utilized in which cell death is mediated by

internalization-dependent delivery of antibody−toxin conjugates. HeLa-CDCP1 and PC-hi/diss cells were incubated with preformed complexes of individual primary antibodies (human scFv-Fc fusion and/or murine mAbs) and a corresponding species-specific saporin-conjugated secondary antibody. Reduced cell viability indicates antibody internalization and successful saporin delivery. As shown in Figure 6, the anti-transferrin receptor (TfR) antibody[23] induced significant cell death as expressed as remaining cell viability (10% remaining). Therefore, anti-TfR served as a positive control for antibody internalization since both HeLa and PC-3 parental cells express the transferrin receptor protein.[24,25] As expected, no cell toxicity was observed with the primary control antibodies, normal mouse IgG or human A4Fc; nor was toxicity seen with toxin-conjugated secondary antibodies in the absence of any primary antibody (Figure 6). In contrast, treatment with mAb 41-2 and C20Fc resulted in a substantial increase of toxin-mediated cell death of HeLa-CDCP1 cells (40% and 50% respectively). The viability of PC-hi/diss cells was reduced to a lesser extent (80% by mAb 41-2 and 70% by C20Fc). Importantly, neither mAb 41-2 nor C20Fc affected viability of HeLa-neo cells, indicative of specific internalization of the anti-CDCP1−CDCP1 ligand complexes by CDCP1-expressing tumor cells.

Discussion

CDCP1 is a highly overexpressed gene that encodes a putative transmembrane protein, containing three CUB domains in the extracellular part in human colon cancer and lung cancer, that was discovered using representational difference analysis and cDNA chip technology in 2001.[1] Indeed, overexpression of CDCP1 has been reported to occur in human colorectal cancer, lung, and renal cell carcinoma and breast cancer.[1,5,9,26] From a biochemical standpoint CDCP1 is a substrate of SRC kinase with Tyr734 becoming phosphorylated;[2,17,18] while phosphorylated CDCP1 is associated within a complex with SRC kinase and PKCδ. Of note is that the tyrosine-phosphorylated CDCP1 is required to overcome anoikis in lung cancer cells.[4]

A particularly intriguing paper by Awakura and colleagues provided strong evidence that expression of CDCP1 in renal cell carcinoma inversely correlated with disease-specific and recurrence-free survivals by microarray, quantitative real-time PCR and immunohistochemical analysis for renal cell carcinoma specimens.[9] Additionally, Ikeda and Oda also reported that high expression of CDCP1 correlates well with relapse rate, poor prognosis, and occurrence of lymph node metastasis.[13]

It has been suggested that CDCP1 plays an important role in tumor growth and survival; indeed the prognosis of patients with overexpressing CDCP1 tumors is much poorer

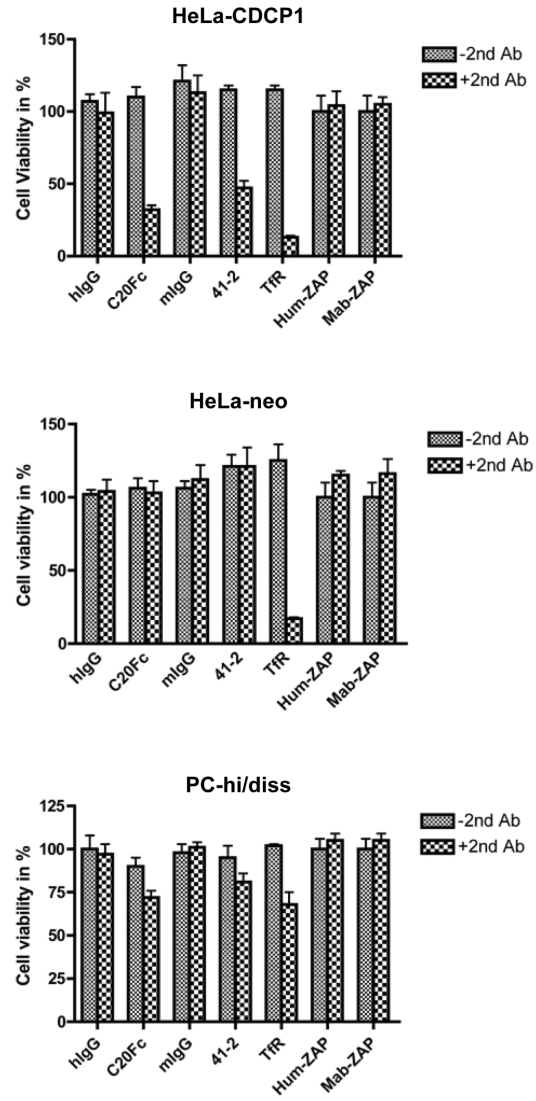

Figure 6. CDCP1 antibody-mediated internalization and tumor cell killing. Internalization was studied using anti-CDCP1 antibodies with an appropriate saporin secondary conjugate for indirect immunotoxin experiments. A PBS vehicle control without antibody served as blank. Preincubated 40 ng primary antibodies and 40 ng/well of goat antimouse saporin (mAb-ZAP) or antihuman secondary saporin (Hum-ZAP) conjugates were added to each well. Percent cell viability compared to nontreated control was determined for primary antibodies with appropriate secondary saporin conjugate. Experiments were done in triplicate over three plates, and a representative graph for the study is shown.

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than that of patients with CDCP1 negative or low expressing tumors.[16] Clinical cancer specimens and cell-based in vitro and in vivo observations strongly suggest that CDCP1 is an excellent target for the development of therapeutic antibodies in cancer therapy.[16] In this regard, generation of fully human anti-CDCP1 antibodies with function blocking capabilities could represent new powerful tools for targeted cancer therapy. In our study, we panned human antibody phage libraries CDCP1 expressing cell lines and immunopurified CDCP1, with the intent of identifying human antibodies that prevent metastasis in vivo. Notably, we used naive antibody phage libraries as unbiased affinity repertoire to select for CDCP1-targeting scFvs. Applying this tactic, a panel of targeting scFvs was identified, including three scFvs that were further characterized. By varying the selection target between cell-bound CDCP1 and immunopurified CDCP1, we should be able to select antibodies targeting different epitopes, thereby broadening therapeutic applicability. All identified scFvs bound specifically to CDCP1-expressing HeLa cells, and one of the identified antibodies was shown to inhibit tumor metastasis in chick embryos as well as in an in vivo mouse model. These highly desirable properties make human anti-CDCP1 antibodies attractive candidates for further therapeutic development.

One of the goals of this study was to test the efficacy of our antibodies in vivo, however, the rapid blood clearance of monovalent scFvs due to their small size can be a limiting factor for the use of these molecules in passive immunotherapy. Bivalent molecules containing the Fc region are more effective at stimulation of Fc-mediated effector functions and have an increased serum half-life in vivo.[27] To exploit these effector functions and to optimize the pharmacokinetic properties of our antibodies, selected scFvs were converted to scFv-Fc fusion proteins. The Fc expression vector used in these experiments, pSec-Fc, contains the hinge and the human IgG1 Fc region. Notably, antibody engineering of the Fc domain allows for the fine-tuning of effector functions and physiological half-life. Because of the efficacy observed of these human anti-CDCP1 scFv-Fc antibodies, future studies using engineered antibodies are warranted and will further accentuate the biological activities against metastatic malignant disease.

Previously, Siva and colleagues identified the murine anti-CDCP1 antibody 25A11 by whole cell panning and subtraction with red blood cells.[8] This antibody inhibited tumor cell migration and invasion in vitro, and the targeted cell surface CDCP1 was internalized together with the 25A11 antibody. When 25A11 was coupled to the cytotoxin saporin, either directly or via a secondary antibody, this toxin conjugate caused killing of CDCP1-positive prostate cancer PC-3 cells in vitro; not surprisingly, the anti-CDCP1 immunotoxin also inhibited primary tumor growth and metastasis of PC-3 cells in a mouse xenograft model. Interestingly, 25A11 without toxin failed to show any inhibitory effects for tumor metastasis.[7] In sharp contrast, our human anti-CDCP1 antibody C20Fc inhibited in vivo tumor metastasis and in vitro colony formation without toxin conjugation. At this juncture the mechanistic difference between these two antibodies remains to be explored in future studies.

One promising area of antineoplastic drug development is the exploration of tumor susceptibility to targeted therapy.[28] Antibodies selectively targeting internalizing epitopes could be harnessed to achieve specific intracellular delivery of therapeutic agents.[29] Two of the identified scFvs were shown to be capable of targeted intracellular payload delivery into CDCP1-expressing cells.

In view of the fact that saporin alone shows low toxicity toward whole cells, we utilized the Hum-Zap assay to prove that, indeed, C20Fc was capable of internalizing into HeLa-CDCP1 and PC-hi/diss cells. The number of viable cells decreased almost 70% for HeLa-CDCP1 cells and 30% for PC-hi/diss cells when treated with equal amounts of Hum-ZAP and C20Fc. While the anti-CDCP1 mAb 41-2, obtained previously from subtractive immunization, also displayed internalization into CDCP1-positive cells as well as inhibited in vivo tumor metastasis,[2] it is important to note that 41-2 is a fully murine immunoglobulin, which limits its clinical applicability.

Over the years there has been a steady increase in our understanding of CDCP1 and its involvement in cancer proliferation. Nevertheless, a number of critical aspects of CDCP1-mediated physiology remain unanswered. The most critical might be to identify the natural ligand(s) and to establish functional roles of CDCP1, which is needed to advance our understanding how CDCP1 interactions with other proteins and processing of CDCP1 impact cell signaling in cancer and normal physiology.

In this report, we demonstrate that the human anti-CDCP1 antibody C20Fc was effective in inhibiting colony formation, thereby underlining the important role of CDCP1 in cancer progression and metastasis and indicating its potential in drug development. Although these data provide an early proof-of-concept for inhibition of tumor metastasis, our findings demonstrate that fully human anti-CDCP1 antibodies can achieve the same feat in inhibiting tumor cell dissemination.

Abbreviations Used

CDCP1, CUB domain containing protein 1; scFv, single-chain variable fragment; mAb, monoclonal antibody; MSC, mesenchymal stem cell; NPC, neural progenitor cell; PKCδ, protein kinase C delta; DMEM, Dulbecco's modified Eagle's medium; IgG, immunoglobulin G; FITC, fluorescein

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isothiocyanate; CAM, chorioallantoic membrane; NOD-SCID, nonobese diabetic severe combined immunodeficiency; LMA, low melting point agarose; CDR, complementarity determining region; scFv-Fc; C-terminal Fc-fused antibodies; SB, super broth; HRP, horseradish peroxidase; FBS, fetal bovine serum; PBS, phosphate buffered saline.

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