Lymphocyte Activation Antigen CD70 Expressed by Renal Cell Carcinoma Is a Potential Therapeutic Target for Anti-CD70 Antibody-Drug Conjugates


Abstract
Metastatic renal cell carcinoma (RCC) is an aggressive disease refractory to most existing therapeutic modalities. Identifying new markers for disease progression and drug targets for RCC will benefit this unmet medical need. We report a subset of clear cell and papillary cell RCC aberrantly expressing the lymphocyte activation marker CD70, a member of the tumor necrosis factor superfamily. Importantly, CD70 expression was found to be maintained at the metastatic sites of RCC. Anti-CD70 antibody-drug conjugates (ADC) consisting of auristatin phenylalanine phenylenediamine (AFP) or monomethyl auristatin phenylalanine (MMAF), two novel derivatives of the anti-tubulin agent auristatin, mediated potent antigen-dependent cytotoxicity in CD70-expressing RCC cells. Cytotoxic activity of these anti-CD70 ADCs was associated with their internalization and subcellular trafficking through the endosomal-lysosomal pathway, disruption of cellular microtubule network, and G2-M phase cell cycle arrest. The efficiency of drug delivery using anti-CD70 as vehicle was illustrated by the much enhanced cytotoxicity of antibody-conjugated MMAF compared with free MMAF. Hence, ADCs targeted to CD70 can selectively recognize RCC, internalize, and reach the appropriate subcellular compartment(s) for drug release and tumor cell killing. In vitro cytotoxicity of these ADCs was confirmed in xenograft models using RCC cell lines. Our findings provide evidence that CD70 is an attractive target for antibody-based therapeutics against metastatic RCC and suggest that anti-CD70 ADCs can provide a new treatment approach for advanced RCC patients who currently have no chemotherapeutic options. (Cancer Res 2006; 66(4): 2328-37)

Introduction
Renal cell carcinoma (RCC) makes up for about 80% of kidney malignancies and 2.6% of all adult cancers. In the United States, ~35,000 patients per year are diagnosed with RCC, with an estimated mortality rate of 13,000 per year (1). The incidence rate approaches 200,000 per year worldwide. Hallmark features of RCC include a characteristically unpredictable clinical course and a 5-year overall survival rate of about 60% (2). Surgical resection of the involved kidney is by far the most successful intervention for localized RCC, yet at diagnosis, nearly 30% of patients present with metastatic disease (3). Despite intense surgical efforts, ~30% to 40% of patients with no evidence of metastasis at time of surgery will subsequently develop distant metastasis (4, 5). Eventually, 50% to 60% of all RCC patients develop metastatic disease (6). The 5-year survival rate for RCC patients with stage IV metastatic disease is a dismal 20% (7). Current methods for surveillance of disease progression after surgery are based entirely on clinical and pathologic indices and do not incorporate molecular markers from tumor tissues (5, 8, 9).

IFN-α and interleukin-2 (IL-2) have been extensively examined for their efficacy against advanced RCC. IFN-α has shown excellent safety profile and can be administered on an outpatient basis. Although overall response rate to IFN-α is limited to 10% to 15%, recent studies have suggested that it may improve patient survival (10, 11). High-dose bolus IL-2 is the only Food and Drug Administration–approved systemic treatment for advanced, unresectable RCC (12, 13). Considerable toxicity, low response rate (<20%), and short duration of response restrict the use of IL-2 to a small subset of patients specifically selected for excellent organ functions. In reality, limited options exist for RCC patients diagnosed with metastatic disease. Recent clinical studies on several classes of experimental drugs, including small molecule inhibitors of protein kinases (14–16) and antibodies targeting vascular endothelial growth factor (17) or the RCC-associated antigen CAIX (18), have shown promise. Continual efforts in the identification of molecular tumor markers that correlate with the risk of developing distant metastasis has the potential to not only improve our ability to predict which patients will progress but also inform on targets for novel therapeutic approaches.

CD70 is a type II integral membrane protein and a member of the tumor necrosis factor (TNF) superfamily transiently expressed on nascent antigen-activated T and B lymphocytes (19). The only known normal, nonlymphoid tissues expressing CD70 are stromal cells of the thymic medulla (20) and mature dendritic cells (21). The receptor for CD70 is CD27, a member of the TNF receptor superfamily (19) expressed by thymocytes, natural killer, T, and B cells (22, 23). Within the lymphoid system, CD70-CD27 interactions regulate T- and B-lymphocyte functions (19, 24, 25) and may be involved in maintaining CD8 memory T-cell responses against viral infections (26–28) as well as allograft rejection mediated by CD8 cells (29).
CD70 was recently reported as a biomarker for RCC during the course of this study (30, 31). Here, we confirm this observation by showing that whereas most normal tissues are CD70 negative, CD70 is aberrantly expressed in both clear cell and papillary RCC. More importantly, CD70 expression persists during metastasis. We also show that antibody-drug conjugates (ADC) consisting of an anti-CD70 monoclonal antibody (mAb) conjugated to novel derivatives of the microtubule disrupting drug auristatin exhibit potent and target-specific in vitro cytotoxicity on CD70-positive RCC cell lines as well as in vivo antitumor activity. Our data suggest that CD70 is an attractive target for antibody-based cytotoxic drug delivery for the treatment of RCC.

Materials and Methods

Cells, antibodies, RNA, cDNA, and frozen tumor sections. RCC lines were obtained from either the American Type Culture Collection (Manassas, VA) or the DSMZ (Braunschweig, Germany). SK-Rc-6 and SK-Rc-7 have been reported previously (32). Anti-CD70 mAb 2F2 and IF6 binding to overlapping epitopes of human CD70 were provided by Dr. Rene van Lier (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands; refs. 22, 33). A chimeric antibody, c1F6, based on the Vκ and Vλ domains of IF6 and human IgG1 isotype was constructed. Reconstituted c1F6 was expressed in Chinese hamster ovary cells and purified by protein A chromatography. Human RCC tumor and normal kidney cDNA were purchased from BioChain Institute (Hayward, CA). Fresh frozen RCC tumors were obtained from Bio RESEARCH Support (Boca Raton, FL). Staged frozen RCC tumor sections were obtained under patient consent and Institutional Board Review approval from the M.D. Anderson Cancer Center (Houston, TX). Frozen RCC tissue microarrays (TMA) were obtained from TriStar Technology Group (Rockville, MD).

Patient cancer profiling array. A cDNA corresponding to nucleotides 734 to 874 of the CD70 mRNA was amplified by PCR using Ramos cell cDNA, the forward primer CD70-734R (5'-CAATGCCTTCTCTTGTCC-3') and the reverse primer CD70-874F (5'-CAATGCCTTCTCTTGTCC-3'). This cDNA was labeled with 32P-dGTP using random hexamers and Klenow fragment (New England Biolabs, Beverly, MA). Purified probe by was hybridized to a Cancer Profiling Array I (BD Biosciences Clontech, Palo Alto, CA) using the BD ExpressHyb hybridization solution (BD Biosciences). Hybridization signals were quantified on a PhosphoImager SI (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunohistochemistry. Frozen tissue sections (large sections/TMA sections) were thawed and air-dried for 2 hours, fixed in acetone for 10 minutes at −20°C, air-dried for 30 minutes, and rehydrated in PBS. Endogenous peroxidase activity was blocked using 0.6% H2O2 for 15 minutes followed by PBS wash. In studies using biotinylated antibodies, slides were treated with successive 30-minute avidin block (Vector Laboratories, Burlingame, CA) and biotin block (Vector Laboratories), Anti-CD70 mAb 2F2, biotinylated 2F2, control nonbinding IgG (MOPC21), or biotinylated MOPC21 was used at a final concentration of 2 μg/mL. Antibody incubation was carried out for 18 hours at 4°C. After PBS washes, slides were incubated with avidin-biotin complex reagent (Elite kit, Vector Laboratories), and 3,3′-diaminobenzidine reagent (Vector Laboratories) was used as peroxidase substrate for color development. Slides were counterstained with hematoxylin, dehydrated, mounted in VectorMount (Vector Laboratories), and observed by light microscopy. Specimens that showed >25% of tumor cells with positive stain by anti-CD70 were considered positive, and specimens with either no staining or <25% of tumor cells stained positive were considered negative.

Immunocytochemistry. 786-O cells (10,000 per well) were attached overnight in Nunc Lab-Tek IV 4-well chambered slides (Fisher, Pittsburgh, PA). After treatment with mAb or ADCs at 1 μg/mL of ADCs or free AFP at 10 nmol/L, cells were washed with PBS, fixed, and permeabilized with the Cytoperm/Cytofix Kit (BD Biosciences). Saturating antibody concentrations and incubation on ice were used in all staining steps. Tubulin was stained using a biotinylated mouse anti-bovine α-tubulin (Molecular Probes, Eugene, OR) followed by Alexa Fluor 488–conjugated streptavidin. For subcellular localization studies, IF6 ADCs were detected by Alexa Fluor 568–conjugated goat anti-human IgG (H+L) with minimal cross-reactivity to mouse IgG (Molecular Probes). Organelles were visualized with mouse antibodies raised against caveolin-1 (clathrin-coated pits), EEA-1 (early endosome marker), Lamp-1 (lysosomal marker), and p290 (trans Golgi marker) from BD Biosciences followed by Alexa Fluor 488–conjugated goat anti-mouse IgG (H+L) with minimal cross-reactivity to human IgG (Molecular Probes). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole at 1 μg/mL in PBS for 10 minutes at room temperature. Cells were mounted with an antifade mounting medium before fluorescence microscopy.

Flow cytometry, cell cycle analysis, and apoptosis assay. For flow cytometry, 2 × 106 cells were incubated in mAb-containing staining medium (RPMI 1640, 5-10% fetal bovine serum) on ice for 20 to 30 minutes. Cells were washed, counterstained with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), washed, fixed in PBS containing 1% of parafomaldehyde, and analyzed on a FACScan (BD Biosciences). Cell surface CD70 receptor copy numbers were determined by flow cytometry using the QFELIKIT (DakoCytomation, Carpinteria, CA). Cell cycle was analyzed by the bromodeoxyuridine/propidium iodide labeling method as previously described (34) and cellular apoptosis was detected using the Annexin V–FITC Apoptosis Detection kit (Oncogene, La Jolla, CA).

Antibody-drug conjugates. Maleimide-caproyl valine-citrulline auristatin phenylalanine phenylendiamine (vcAFP) and maleimide-caproyl valine-citrulline monomethyl auristatin phenylalanine (vcMMAF) were synthesized and conjugated onto antibodies as reported before (35, 36). A mean number of 4 or 8 drug molecules were conjugated to one molecule of IF6 to yield IF6-vcAFP and IF6-vcMMAF conjugates. ADCs used in this study typically contained <2% protein aggregates and <0.5% unconjugated free drug.

Proliferation inhibitory and cytotoxicity assays. Cells (1,000-3,000 per well) in 100 μL of medium in 96-well flat-bottomed plates were incubated overnight. An additional 100 μL of culture medium with varying concentrations of ADCs were added to quadruplicate wells, and incubation was continued for an additional 96 hours. DNA synthesis was assayed by 3H-thymidine incorporation during the last 16 hours of incubation. Cytotoxicity was assayed by Alamar Blue (Biosource International, Camarillo, CA) reduction. Dye reduction was measured by fluorescence spectrometry at excitation and emission wavelengths of 535 and 590 nm, respectively.

In vivo xenograft tumor model of RCC. Caki-1 or 786-O tumor tissue blocks of ~30 mm3 were prepared under aseptic conditions from tumor-bearing donor mice. Naive nude mice were each implanted s.c. with one tumor block of 30 mm3. Treatment was initiated when the average tumor size within a group reached ~100 mm3. Treatment consisted of i.v. injections of mAb or ADCs either as a single administration or every 4 days for four injections (q4dx4). Tumor dimensions were determined by caliper measurements, and tumor size was calculated using the formula of (length x width2) / 2. Mice were euthanized as tumor size reached 1,000 mm3. All xenograft experiments were conducted under Institutional Animal Care and Use Committee guidelines and approval.

Statistics. Statistical analysis was conducted using R × C × 2 contingency tables and Fisher's exact test for immunohistochemistry studies and Student's t test for xenograft tumor model studies.

Results

CD70 transcript expression in RCC. Screening of a cancer profiling array (CPA) revealed that expression of CD70 transcripts in 9 of 20 (45%) cases of kidney carcinoma, two cases of stomach carcinoma, and one case each of breast, colon, rectum, and ovary carcinoma was 2-fold than the matched normal tissues.
Quantitative PCR analysis was conducted on seven additional, independent cases of clear cell RCC, of which six overexpressed CD70 transcripts (data not shown), confirming the results obtained from CPA analysis. Similar size CD70 transcripts were detected in both RCC and hematopoietic cells, and cDNA clones isolated from RCC cells also had identical nucleotide sequence to the published CD70 cDNA sequence (data not shown).

CD70 protein expression in RCC. Immunohistochemical analysis showed specific anti-CD70 mAb binding to RCC on tissue sections (Fig. 1B). Staining was associated with cell membrane and cytoplasm but not stromal tissues and was homogeneous within tumor-containing areas. Anti-CD70 mAb did not bind to adjacent normal tissues of the same RCC samples, suggesting minimal or no CD70 protein expression in normal kidneys. CD70 protein expression was not detectable in the following normal tissues tested: heart, lung, liver, cerebrum, cerebellum, bladder, stomach, colon, mammary gland, adrenal gland, thyroid gland, prostate gland, placenta, uterus, fallopian tubule, ovary, skin, testis, and muscle. Immunohistochemical staining using the anti-CD70 antibody 1F6 also showed concordant results.

Figure 1. Differential CD70 transcript and protein expression in RCC. A, CD70 transcript expression was examined in a patient CPA as described in Materials and Methods, because equivalent cDNA loading was confirmed by probing the same array with the housekeeping EF-1 gene, ratios between CD70 hybridization signals obtained from tumor-derived and corresponding normal tissue-derived cDNAs were used to compare CD70 transcript expression among different cancer types. Each dot represents the CD70 tumor/normal expression from one patient isolate. CD70 expression in cancer types where six or more matched cDNA pairs were available was included in this analysis. A tumor/normal ratio of 2-fold increase was used as the arbitrary cutoff for CD70 overexpression (blue line). Red lines indicate the mean tumor/normal ratios. B, binding of the anti-CD70 mAb 2F2 and a control IgG to fresh frozen tumor sections derived from two RCC donors was determined by immunohistochemistry staining (left). Photomicrographs were taken under ×40 magnification. The same pair of antibodies was used to stain frozen sections from adjacent normal kidneys of the same RCC donors used in (right). C, CD70 expression at multiple tumor sites from a stage IV patient (Pt. 1), including the primary tumor, inferior vena cava tumor thrombus, and lymph node metastasis, and from the bone metastasis of a second stage IV patient (Pt. 2).

Table 1. CD70 expression in RCC

<table>
<thead>
<tr>
<th>AJCC staging</th>
<th>Fuhrman nuclear grading</th>
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<tbody>
<tr>
<td></td>
<td>Large tissue section analysis</td>
</tr>
<tr>
<td>Clear cell</td>
<td>n = 40</td>
</tr>
<tr>
<td>I</td>
<td>0/10⁸ (0)</td>
</tr>
<tr>
<td>II</td>
<td>1/10 (10)</td>
</tr>
<tr>
<td>III</td>
<td>3/10 (30)</td>
</tr>
<tr>
<td>IV</td>
<td>8/10⁸ (80)</td>
</tr>
<tr>
<td>Papillary</td>
<td>n = 23</td>
</tr>
<tr>
<td>I</td>
<td>NA¹</td>
</tr>
<tr>
<td>II</td>
<td>NA</td>
</tr>
<tr>
<td>III</td>
<td>NA</td>
</tr>
<tr>
<td>IV</td>
<td>NA</td>
</tr>
<tr>
<td>Chromophobe</td>
<td>n = 6</td>
</tr>
<tr>
<td>I</td>
<td>NA</td>
</tr>
<tr>
<td>II</td>
<td>NA</td>
</tr>
<tr>
<td>III</td>
<td>NA</td>
</tr>
<tr>
<td>IV</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.
* n = number of independent patient isolates available.
¹ Number of CD70-positive cases/total number of cases.
²% CD70 positive.
³ V P ≤ 0.0055 by Fisher’s exact test when compared with stage I or II in large section analysis.
⁴Tumor specimens were not available.

More than 160 cases of RCC of different histologic subtypes were analyzed to explore potential relationship between CD70 expression and disease status. Two independent immunohistochemical studies were done, one on 40 large tissue sections and another one on TMA sections containing 122 tumors (Table 1). All specimens were categorized using the American Joint Cancer Committee (AJCC) staging. In our large section study, 40 cases of clear cell RCC with Fuhrman nuclear grades of 3 to 4, each with matched normal kidney sections, were individually processed for immunohistochemical staining. For the 10 stage IV samples, matched normal kidney sections, were individually processed for immunohistochemical staining. For the 10 stage IV samples, matched tissues from metastatic sites were also available. Remarkably, metastatic tumors for the eight CD70-positive stage IV samples from metastatic sites were also available.

FIGURE 1. Immunohistochemical staining for CD70 in clear cell RCC. Immunohistochemical analysis of the large scale study revealed that CD70 is frequently but not always expressed in clear cell RCC in stages I-IV (70%), II (60%), III (70%), and IV (50%). In contrast, papillary RCC express CD70 in stages I, II, and III (20%, 40%, and 80%, respectively). No correlation between CD70 expression to AJCC staging or Fuhrman nuclear morphology was observed in either clear cell or papillary RCC in this larger scale study.

Flow cytometry confirmed CD70 protein expression on RCC surface. Of 10 RCC lines tested, none were CD70 positive, with receptor copy numbers ranging from 8,000 to >500,000 (Table 2). In contrast to immunohistochemical staining on normal kidney where CD70 was shown to be consistently absent, cultured normal renal proximal tubule epithelial and human renal cortical epithelial cells (RPTEC and HRCE) expressed low but detectable surface CD70. It is therefore possible that propagation of normal renal epithelial cells might have induced CD70 expression in vitro.

Anti-CD70 antibody-drug conjugates. Auristatin E is a microtubule disrupting agent, and ADCs consisting of the monomethyl derivative of auristatin E, MMAE, have potent and target-specific cytotoxicity against tumors expressing the CD30 receptor copy numbers ranging from 8,000 to >500,000 (Table 2). Two new auristatins, AFP (35) and MMAF (36), were examined in this study.

Using a vc dipeptide linker and the maleimidocaproyl group for coupling, AFP or MMAF was conjugated to the anti-CD70 mAb murine 1F6 (m1F6) or its chimeric (c) derivative c1F6 (Fig. 2A and B). Binding of these ADCs to CD70-expressing cells was similar to unconjugated 1F6 (Fig. 2A and B, right). These conjugates also completely cross-blocked 1F6 binding (data not shown), confirming that drug conjugation did not alter binding specificity. In this study, we examined 1F6 ADCs containing either 4 or 8 molar equivalent of drug molecules, as indicated in the figures and Table 2.

In vitro proliferation inhibitory and cytotoxic effects of 1F6 ADCs. m1F6-vcAFP inhibited the proliferation of the
CD70-positive RCC lines 786-O and Caki-1 dose dependently (Fig. 2C), with a concomitant loss in cell viability (Fig. 2D). c1F6-vcMMAF was cytotoxic against both 786-O and Caki-1 cells with similar efficacy, suggesting that both conjugation approaches resulted in ADCs of comparable potencies (Fig. 2E). 1F6 ADCs were at least 1,000-fold more potent against CD70-positive cells compared with the nonbinding control IgG-vcAFP, showing their target antigen specificity (Fig. 2A and B). The selectivity of 1F6 ADCs was supported by the much higher IC50 values for proliferation inhibition and cytotoxicity on CD70-negative cell lines Hs835.T (Fig. 2E; Table 2) and Jurkat (data not shown).

The in vitro antitumor activity of 1F6-vcAFP, 1F6-vcMMAF, and free MMAF against a panel of RCC cell lines and normal renal epithelial cell culture is summarized in Table 2. This panel consisted of cell lines derived from clear cell and papillary RCC and from primary tumors, skin metastasis, or pleural effusion. The IC50 values for 1F6 ADC-mediated proliferation inhibition ranged from 1 to 247 ng/mL for CD70-positive RCC lines. Similar IC50 values were obtained in cytotoxicity assay using Caki-1, Caki-2, 786-O, A498, and CAL54 cells. At these ADC concentrations, the molar concentration of MMAF delivered by 1F6 ranged from 0.1 to 37 nmol/L (Table 2), considerably lower than the concentrations of free MMAF needed to achieve equivalent cell killing (Table 2).

Despite detectable CD70 expression in the normal kidney epithelial cultures RPTEC and HRCE, substantially higher doses of 1F6-vcAFP were needed to inhibit proliferation. In addition, 1F6 ADCs was nontoxic to RPTEC and HRCE cells at concentrations as high as 10 μg/mL (Fig. 2F; Table 2). This differential response was not due to differential sensitivity of cells toward the 1F6-delivered AFP or MMAF, as RPTEC and HRCE cells were comparably sensitive to free AFP and MMAF as 1F6-negative RCC lines (data not shown).

1F6 ADC-mediated disruption of microtubule network, cell cycle arrest, and apoptosis. Subcellular localization of 1F6 ADCs in RCC cells was examined by fluorescence microscopy. Figure 3A shows a representative experiment conducted with

### Table 2. Expression of CD70 by RCC lines and their response to 1F6 ADCs

<table>
<thead>
<tr>
<th>CD70 copy number* (x(10^{-3}))</th>
<th>Proliferation inhibition(^{1}) (IC50, ng/mL)</th>
<th>Cytotoxicity(^{1}) (IC50, ng/mL)</th>
<th>Drug delivered at IC50 of m1F6-vcMMAF(^{2}) (nmol/L)</th>
<th>IC50 of free MMAF (nmol/L)(^{1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caki-1, skin metastasis</td>
<td>136</td>
<td>9</td>
<td>&gt;2,000</td>
<td>24</td>
</tr>
<tr>
<td>Caki-2, primary tumor</td>
<td>170</td>
<td>2</td>
<td>1,150</td>
<td>3</td>
</tr>
<tr>
<td>786-O, primary tumor</td>
<td>264</td>
<td>9</td>
<td>&gt;5,000</td>
<td>12</td>
</tr>
<tr>
<td>769-P, primary tumor</td>
<td>8</td>
<td>152</td>
<td>2,500</td>
<td>311</td>
</tr>
<tr>
<td>Papillary</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACHIN, pleural effusion</td>
<td>44</td>
<td>247</td>
<td>&gt;5,000</td>
<td>696</td>
</tr>
<tr>
<td>CAL54, pleural effusion</td>
<td>41</td>
<td>17</td>
<td>ND</td>
<td>21</td>
</tr>
<tr>
<td>A498, primary tumor</td>
<td>503</td>
<td>5</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td>CD70 negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hs835.T, primary tumor</td>
<td>0.3</td>
<td>1,167</td>
<td>1,250</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>Primary renal epithelium</td>
<td></td>
<td></td>
<td></td>
<td>&gt;50</td>
</tr>
<tr>
<td>RPTEC</td>
<td>19</td>
<td>325</td>
<td>ND</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>HRCE</td>
<td>5</td>
<td>1,150</td>
<td>ND</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

*CD70 copy number was determined by quantitative flow cytometry as described in the Materials and Methods.

\(^{1}\)Proliferation was assayed by \(^{3}\)H-thymidine incorporation.

\(^{2}\)Cytotoxicity was assayed by reduction of Alamar Blue.

\(^{2}\)Molar concentration of MMAF delivered by m1F6-vcMMAF at its IC50 determined by Alamar Blue dye reduction.

\(^{1}\)IC50 of free MMAF was determined by Alamar Blue dye reduction.
Anti-CD70 Drug Conjugates Targeting RCC

**Figure A**

![Diagram of 1F6-vcAFP conjugate]

**Figure B**

![Diagram of c1F6-vcMMAF conjugate]

**Figure C**

![Graphs showing 3H incorporation and viability of 786-O, m1F6-vcAFP, Caki-1, m1F6-vcAFP, and Caki-1, IgG-vcAFP conjugates]

**Figure D**

![Graphs showing viability of 786-O, m1F6-vcAFP, Caki-1, m1F6-vcAFP, and Caki-1, IgG-vcAFP conjugates]

**Figure E**

![Graphs showing viability of 786-O, c1F6-vcMMAF4, Caki-1, c1F6-vcMMAF4, and Hs835T, c1F6-vcMMAF4 conjugates]

**Figure F**

![Graphs showing viability of RPTEC, m1F6-vcAFP, HRCE, m1F6-vcAFP, and HRCE, m1F6-vcMMAF conjugates]
786-O cells. 1F6-vcMMAF was colocalized with clathrin, early endosome, and lysosome as early as 4 hours after ADC treatment and was maintained 10 hours after ADC treatment. Similar 1F6 ADC subcellular trafficking route was also observed in Caki-1 cells (data not shown). Specific localization of 1F6 ADC to the endosomal-lysosomal pathway was suggested by the lack of colocalization with the trans-Golgi apparatus. Although the nonbinding IgG control ADC had little effect on microtubule network, treatment of RCC lines with AFP, 1F6-vcAFP, or 1F6-vcMMAF for 8 hours induced complete disruption of microtubule network (Fig. 3B; data not shown). This disruption of the microtubule network was accompanied by cell cycle arrest (Fig. 3C). A progressive G2-M phase arrest preceded the appearance of apoptotic events characterized by DNA fragmentation in 1F6-vcAFP–treated cells. Neither 1F6 mAb alone nor IgG-vcAFP exerted discernible cell cycle arrest in 786-O cells compared with medium only control cells, but significant cell cycle arrest began in 1F6-vcAFP–treated cells as early as 3 hours after drug addition (Fig. 3C). G2-M phase arrest was maximal 12 hours after drug administration followed by the emergence of apoptotic cells. More than 40% apoptotic cells were detected after 24 hours of ADC treatment. Consistent with DNA fragmentation, apoptosis induction was confirmed by the appearance of Annexin V–binding cells in 1F6-vcMMAF–treated cells (Fig. 3D). Taken together, these results suggest that the proliferation-inhibitory and apoptosis-inducing activities of 1F6 ADC was associated with microtubule network destruction and G2-M phase cell cycle arrest.

**In vivo efficacy of 1F6 ADCs in xenograft models of RCC.** Using xenografts of the Caki-1 and 786-O lines in nude mice, we extended the *in vitro* antitumor activity of 1F6 ADCs to *in vivo* models of human RCC. In the absence of any treatment, mean tumor volume increased to ~800 mm³ within 40 days for Caki-1 tumors and ~600 mm³ within 30 to 40 days for 786-O tumors (Fig. 4). Treatment with 1F6 ADCs profoundly suppressed *in vivo* tumor growth in both models. For the Caki-1 model, average tumor size in the 1F6-vcAFP–treated group remained <180 mm³ at the end of the experiment 77 days after tumor implantation, whereas nonbinding control IgG-vcAFP did not show any antitumor activity (Fig. 4A). Several dosage and schedule combinations were further examined using the 786-O model. When used at graded concentrations, 1F6-vcMMAF doses as low as 1 mg/kg on a q4dx4 schedule showed significant activity in delaying tumor growth (Fig. 4B). One hundred and 75% of the animals treated with 3 and 1 mg/kg of 1F6-vcMMAF, respectively, achieved complete remission as defined by the absence of palpable tumor at site of implantation. Cytotoxic drug delivery was required for *in vivo* efficacy, as unconjugated c1F6 at 10 mg/kg did not show any detectable antitumor activity in this setting (Fig. 4B). Targeting specificity in the 786-O model was also confirmed by the lack of activity with a nonbinding IgG-vcMMAF conjugate (data not shown). A single administration of 1F6-vcMMAF at either 0.75 or 1.5 mg/kg was able to significantly delay 786-O tumor growth, highlighting the *in vivo* potency of 1F6 ADC (Fig. 4C).

**Discussion**

To maximize targeting specificity and minimize toxicity of experimental drugs, abundant tumor expression but low or absence of expression in the corresponding normal tissue and other vital organs, uniform expression throughout primary tumor and corresponding metastases, high percentage of patients with tumor displaying antigen, and minimal antigen shedding are much favored features (39). Receptors transiently expressed by activated lymphocytes but not by other normal tissues would be attractive candidates for therapeutic targeting if they are also expressed on the surface of transformed cells. The IL-2 receptor complex expressed on activated lymphocytes is an example in which denileukin diftitoxin (ONTAK; refs. 40, 41) uses IL-2 for toxin delivery to target cells. Denileukin diftitoxin has shown clinical efficacy in patients with IL-2 receptor–expressing persistent or recurrent cutaneous T-cell lymphoma (40, 41).

We report aberrant expression of the lymphocyte activation antigen CD70 in 30% to 68% of clear cell RCC and 38% to 40% of papillary RCC, the two most common histologic subtypes of RCC (Table 1). These percentages are consistent with the transcript-based CPA analysis (Fig. 1A). CD70 overexpression has recently been shown in a metastatic subclone of the breast carcinoma line MDA-MB-435, suggesting that it may potentially be a metastasis-related gene (42). Analysis of >160 primary RCC isolates has not revealed a clear correlation between CD70 expression and disease stage or nuclear morphology (Table 1). However, where matched metastatic lesions were available for the stage IV patient isolates, CD70 was detected in the metastatic sites whenever there was CD70 expression in the primary tumor (Table 1; Fig. 1C). These results suggest that a positive CD70 status in primary tumors may indicate that the metastases of this tumor could be most likely be amenable for targeting by anti-CD70 drugs. In addition, CD70 may potentially be used as an imaging marker to locate metastatic tumors.

Similar to other solid tumor lesions, tumor-infiltrating T lymphocytes are readily detectable in RCC (43). The potential expression of CD70 on tumor-infiltrating lymphocytes as well as the role of CD70 expressed on RCC cells on host antitumor immune response needs to be further examined. It is noteworthy that in glioblastoma, CD70 expression has been implicated in tumor evasion of immune surveillance (44, 45).

The frequency of CD70 expression observed in this study is discordant with the recent articles, in which 100% of clear cell RCC (30, 31) but only 1 of 19 cases of papillary RCC expressed CD70 (30). This may be due to differences in the immunohistochemical staining methods used as well as the patient populations being sampled. Larger-scale studies using standardized reagents and staining procedures are obviously needed to more accurately define the frequency of CD70 in clear cell and papillary RCC. These conflicting data also clearly imply that targeted anti-CD70 therapy for RCC will need the development of a diagnostic tool to screen patients for tumor-associated CD70 expression.

The anti-CD70 mAb 1F6 was extremely efficient in delivering cytotoxic drugs to the interior of RCC cells. The IC₅₀ values of 1F6 ADC on proliferation inhibition and cytotoxicity were (Fig. 2; Table 2) well below saturation levels for binding, (Fig. 2A and B). Drug delivery was specific as well as selective for CD70 (Table 2). A comparison of IC₅₀ of free MMAF and c1F6-vcMMAF conjugate revealed that the molar concentrations of free MMAF required to elicit 50% killing in the RCC lines examined in this study was at least 10-fold higher than that delivered by 1F6-vcMMAF (Table 2), further illustrating the
efficiency of drug delivery via CD70 targeting. Besides auristatin-based 1F6 conjugates, DNA-binding cytotoxic drugs have also shown potent, target-selective cytotoxic activities (46). This drug delivery capability is also not limited only to mAb 1F6. Other anti-CD70 mAb (e.g., BU69, HNE.51, and 2F2) can also specifically target and kill CD70-positive cells by delivering auristatins or the ribosome-inactivating toxin saporin conjugated onto secondary anti-mouse antibodies (data not shown). In addition to CD70-positive RCC, 1F6 ADCs also show target-specific cytotoxicity against CD70-positive lymphoma cells and activated lymphocytes, suggesting potential applications of 1F6 ADCs in lymphoma treatment and immunosuppression.5

The vc dipeptide linker system is designed to be stable in the extracellular environment, whereas highly susceptible to proteolytic degradation by lysosomal proteases (38). Specific, targeted cytotoxicity of the 1F6 ADCs (Figs. 2 and 4; Table 2) suggests this ADC was internalized by target cells and trafficked through the appropriate intracellular pathway for drug release. Cell-bound 1F6 and 1F6 ADCs undergo temperature-dependent down modulation indicative of receptor-mediated endocytosis (data not shown). Microscopy experiments revealed that internalized 1F6 ADCs trafficked through the endosomal-lysosomal pathway. Endosomal-lysosomal sorting signals are contained within the cytoplasmic domains of transmembrane proteins. One such endosomal-lysosomal sorting signal responsible for clathrin interaction during internalization and lysosomal targeting of transmembrane proteins is the tyrosine-based motif containing

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the amino acid sequence of YXXØ (47). In this motif, X can be any amino acid and Ø is an amino acid with a bulky hydrophobic side chain. The cytoplasmic tail of CD70 contains an YGCV motif at positions 15 to 19 that conforms to this YXXØ consensus. The cytotoxic activity of 1F6 ADCs coupled with the subcellular localization experiments support the model of 1F6 ADC internalization via clathrin-coated pits and trafficking to the lysosomes for ADC degradation and drug release.

Auristatins are synthetic analogues of the natural product dolastatin 10, which interacts with the Vinca alkaloid-binding site on α-tubulin to prevent tubulin polymerization (48–50). ADCs consisting of auristatin E induce G2-M phase arrest in target cells preceding the onset of apoptosis (34, 37). We show that 1F6 ADCs could completely disrupt the microtubule network of target cells. Onset of microtubule disruption correlated with G2-M phase arrest and preceded the burst in apoptosis (Fig. 3B-D). This is consistent with the idea that the cytotoxic effect of 1F6 ADCs was mediated through the intracellular release of free AFP or MMAF.

In xenograft RCC models, single dose administrations of as low as 0.75 mg/kg conferred substantial delay in tumor progression, and complete remission was observed in a subset of treated mice in the 786-O model (Fig. 4C). In a multiple dose schedule, complete remission rate was 75% when c1F6-vcMMAF4 was administered at 1 mg/kg (Fig. 4B). These doses produced no detectable toxic effects in mice, and 1F6-vcMMAF4 could be tolerated in immunocompetent mice at 40- to 50-fold higher than the therapeutic dose used in the 786-O xenograft model. Stromal cells of the thymic medulla (20) and mature dendritic cells (21) aside, CD70 is not known to be expressed on normal, nonhematopoietic cells. Because very few lymphocytes circulating in blood or residing in secondary lymphoid organs are activated and expressing CD70, an advantage of therapeutics targeting CD70 may be limited drug-related toxicity. The lack of detectable binding of anti-CD70 mAbs to normal, nonhematopoietic human tissues also suggests a significant therapeutic window may be achievable by auristatin-based anti-CD70 ADCs. However, studies to critically assess the safety of anti-CD70 ADCs and their effects on the immune system and to establish their side effects both on-target and off-target tissues are certainly required to enable eventual testing of this therapeutic approach in RCC patients. Given that 1F6 does not cross-react with the murine CD70 orthologue, ongoing safety evaluation of 1F6 ADCs are focused in nonhuman primates.

Our data confirm that CD70 is a novel RCC-associated surface antigen and a potential marker for high-grade metastatic disease. We also show that CD70 can be exploited as target for cytotoxic drug delivery to RCC cells. In addition to RCC, other CD70-expressing cancers, such as B lymphoma, nasopharyngeal carcinoma, thymic carcinoma, neuroblastoma, and glioblastoma, may be suitable targets for anti-CD70 ADCs as well. We are currently optimizing an anti-CD70 ADC consisting of the appropriate cytotoxic drug and linker chemistry suitable for clinical development against chemoresistant metastatic RCC.

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