Carbonic anhydrase IX has chaperone-like functions and is an immunoadjuvant

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Abstract

Carbonic anhydrase IX (CA9) is a hypoxia-regulated, transmembrane protein associated with neoplastic growth in a large spectrum of human tumors. CA9 is expressed in nearly all clear-cell renal tumors; levels of CA9 expression predict prognosis and response to interleukin-2 therapy. These observations may be explained by a novel chaperone-like function of CA9, which allows it to serve as an immunoadjuvant and stimulate an adaptive immune response against tumor antigens. Classic heat shock proteins (HSP) such as HSP110 and HSP70 are up-regulated in response to cellular stress and function to protect intracellular proteins from aggregation. Similarly, CA9 formed complexes with client proteins and inhibited heat-induced aggregation and enabled refolding of denatured client protein. HSP released from injured cells activate an immune response. CA9 bound dendritic cells in a receptor-specific manner. Bound CA9 was internalized by dendritic cells and processed primarily through the proteosomal pathway. In a murine melanoma model, a complex of CA9 and gp100 generated a gp100-specific antitumor response. A soluble form of CA9 shed from tumor cells had the same chaperone-like functions, providing renal tumors and hypoxic cells with a mechanism for stimulating an immune response against extracellular antigens. Interleukin-2 treatment of patient renal tumors in short-term culture increased CA9 shedding, suggesting a strategy for augmenting the immunogenicity of renal tumors. CA9 has chaperone-like functions and CA9 shed from tumors may play a direct role in stimulating an adaptive immune response. [Mol Cancer Ther 2008;7(12):3867 – 77]

Introduction

Carbonic anhydrase IX (CA9) expression is associated with neoplastic growth and has been correlated with prognosis in cancers of the brain, lung, breast, cervix, kidney, gastrointestinal track, and head and neck (1–6). CA9 is a transmembrane protein with an extracellular enzyme domain that catalyses the following reaction: $H_2O + CO_2 \leftrightarrow H^+ + HCO_3$. This reaction contributes to the regulation of intracellular pH, providing cells with a survival advantage in an acidic microenvironment (7).

In most cancers, CA9 is a marker for hypoxia. CA9 expression is increased in bulky tumors, with greatest expression in tumor cells immediately adjacent to areas of necrosis (1). However, in clear-cell renal cell carcinoma (RCC), CA9 expression is not regulated by oxygen tension. Instead, CA9 is overexpressed due to inactivation of the von Hippel-Lindau gene product. von Hippel-Lindau normally functions to degrade and suppress hypoxia-inducible factor-α, which is as a transcription factor for CA9 (8, 9).

For clear-cell RCC, CA9 is that "perfect" biomarker that establishes diagnosis (10–13), determines prognosis (5, 14–16), predicts treatment response (17, 18), and serves as a target for therapy (19–22). CA9 is present in >80% of primary and metastatic RCC and is present in 95% to 100% of the clear-cell variant (23). In clear-cell RCC, high CA9 expression was an independent predictor of longer disease-specific survival in patients with metastatic RCC (5, 14) and improved recurrence-free survival in patients with localized RCC (15, 16).

Given the nearly universal and tumor-specific expression of CA9 by clear-cell RCC, CA9 is an attractive target for therapy. An international, phase III clinical trial is currently under way to evaluate a humanized monoclonal antibody against CA9 in patients at high risk for recurrence following surgical resection (24). A variety of additional immunotherapeutic strategies have been described targeting CA9 in animal models (19, 20) and early-phase clinical trials (21, 22).

IFN and interleukin (IL)-2 have been used to treat metastatic RCC for the past 15 years with response rate of 15% to 20% (17). CA9 is the only independently confirmed biomarker for predicting response for any immunoresponsive tumor. Bui et al. first suggested that patients with increased CA9 expression may be more likely to respond to IL-2-based therapy (14). This observation was confirmed by Atkins et al. using pathology specimens from patients treated on IL-2-based RCC trials (18).

Despite the widespread interest in CA9 and better understanding of the molecular defect leading to CA9 overexpression in clear-cell RCC, the mechanism linking CA9 expression to improved prognosis and treatment response has never been elucidated. We describe a novel

chaperon-like property of CA9, which is similar to properties attributed to heat shock proteins (HSP). To the best of our knowledge, this is the first example of a cell surface protein that can function as a chaperone. Like HSPs, CA9 is able to complex antigens and generate an antigen-specific immune response.

Zavada et al. described a soluble form of CA9 that is shed (sCA9) from the surface of tumor cells (25). CA9 shedding has been reported to be a regulated process that is metalloprotease dependent rather than a nonspecific consequence of cell turnover (26). The function of sCA9 has never been identified. We report that sCA9 also has chaperone-like properties and can function as an immunoadjuvant, providing CA9-expressing cells with a mechanism for recruiting the immune system to target extracellular antigens.

Materials and Methods

Mice, Cell Lines, and DNA Constructs

Female C57/BL6 mice, 6 to 8 weeks old, were purchased from National Cancer Institute and housed under pathogen-free conditions. SR-A-null mice were back-bred into the C57BL/6J background and were obtained from B. Berwin (Dartmouth University) as a generous gift of T. Kodama (Tokyo University) and M.W. Freeman (Massachusetts General Hospital, National Heart, Lung, and Blood Institute Program in Genomics Applications). Human gp100-transduced B16 cells (B16-gp100) were kindly provided by Dr. Alexander Rakhmilevich (University of Wisconsin). R6, a human RCC cell line that expresses CA9, was a gift from Dr. Arie Belldegrun (University of California-Los Angeles). RENCA and RENCA cells stably transduced to express human CA9 (RENCA-CA9) were a gift from Dr. Arie Belldegrun. See Supplementary Data[3] for culture conditions.

The cDNA for mouse HSP110, mouse HSP70, human CA9, (a gift from Dr. Arie Belldegrun), and human gp100 (a gift from Dr. Nicholas Restifo, National Cancer Institute) were cloned into pBacPAK-his vector (BD Biosciences Clontech) and recombinant proteins were produced using the BacPAK baculovirus system. See Supplementary Data[3] for details.

Formation of Chaperone-Antigen Complex

To form a complex between chaperone (HSP110, CA9, and ovalbumin) and antigen (gp100 and luciferase), the two proteins were combined at 1:1 molar ratio and incubated for 30 min at 37°C or at heat shock temperatures of 43°C as described previously (27). The complex was pretreated with 30 μL protein G beads and immunoprecipitated using a mouse anti-human CA9 monoclonal antibody (a gift from Dr. Egbert Oosterwijk, University of Nijmegen), a previously described rabbit anti-HSP110 antibody (28), or mouse anti-ovalbumin antibody (Sigma). After 10% SDS-PAGE, Western blot analysis was done

using anti-luciferase (Promega) or anti-gp100 antibody (Santa Cruz Biotechnology).

Luciferase Aggregation and Refolding Assay

For the luciferase aggregation assay, 0.15 μmol/L luciferase (Sigma) and chaperone protein (CA9, HSP110, and HSP70) were incubated in 25 mmol/L HEPES (pH 7.9), 5 mmol/L magnesium acetate, 50 mmol/L KCl, and 5 mmol/L β-mercaptoethanol at 43°C for 30 min. Ovalbumin served as a control for chaperone proteins. Protein aggregation was monitored by measuring absorbance at 320 nm. To confirm that chaperone proteins were preventing aggregation, the solutions were centrifuged at $16,000 \times g$ for 15 min, and soluble and pellet fractions were separated, run on SDS-PAGE, and subjected to Western analysis with anti-luciferase antibody (Promega).

For luciferase refolding assay, luciferase and chaperone protein were heated in refolding buffer [25 mmol/L HEPES (pH 7.6), 5 mmol/L $MgCl_2$, 2 mmol/L DTT, and 2 mmol/L ATP] at 43°C for 30 min. The heated luciferase was diluted 100-fold into refolding buffer containing 60% rabbit reticulocyte lysate (Promega) and incubated at 30°C for 2 h. To measure luciferase activity, the solution was further diluted 5-fold in 25 mmol/L HEPES (pH 7.6) and 1 mg/mL bovine serum albumin (BSA); 10 μL were added to 100 μL luciferase assay solution (Promega). Luciferase activity was quantified using a Lumat LB9501 luminometer (Berthoid).

Tumor Prevention Study

Female C57/BL6 mice (National Cancer Institute), 6 to 8 weeks old (5 per group), were immunized three times, 7 days apart, with 100 μL vaccine. Mice were challenged with 2×10^5 B16-gp100 cells injected i.d., 7 days after the last immunization. Tumors were measured every 3 days using an electronic caliper and tumor volume was calculated [(shortest diameter^2 × longest diameter) / 2]. The complete set of experiments was repeated three times. The ELISPOT and ^{51}Cr release assays have been described (27). See Supplementary Data[3] for a brief description.

Generation of Immune Response with sCA9

Mice (5 per group) were immunized three times, 7 days apart, with dendritic cell-based vaccines. The vaccination groups included dendritic cells treated with a complex of CA9 and murine gp100 peptide (EGSRNQDWL with >99% purity by high-performance liquid chromatography, synthesized by Alpha Diagnostic international): CA9 + peptide, HSP110 + peptide, and sCA9 + peptide. Untreated dendritic cells and ovalbumin + peptide served as negative controls. To form protein-peptide complexes, 2 μg peptide was incubated for 30 min with 20 μg proteins (ovalbumin at 43°C, CA9 at 37°C, sCA9 at 37°C, or HSP110 at 43°C). Peptide-protein complexes were added to bone marrow-derived dendritic cells.

To generate dendritic cells, marrows were harvested from murine femurs and tibias and treated with RBC lysis buffer, washed, and plated at a density of 1×10^6 cells/mL in 12-well plates in RPMI 1640 containing 10% fetal bovine serum and 10 ng/mL recombinant mouse granulocyte-macrophage colony-stimulating factor (eBioscience). Cells

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

were fed every 2 days and harvested between days 7 and 9. Cultures consisted of 75% to 90% CD11C$^+$ cells. To generate vaccines, cultured cells were pulsed for 4 to 6 h with 10 μg/mL protein-peptide complex and treated with 100 ng/mL lipopolysaccharide for 16 h. Cells (2 × 10^6) were injected s.c. into mouse. Seven days after the last immunization, lymph nodes and splenocytes were harvested for *in vivo* and *in vitro* CTL assays.

Response of CA9 to Cytokines

CA9 expression was monitored by probing R6 cell lysates with anti-CA9 antibodies after treating with conditioned medium at 200 μL/mL for 48 h. WBCs were separated from whole blood obtained from healthy human subjects, and culture medium from WBCs treated with 100 ng/mL cytokines or nothing (control) for 24 h served as the conditioned medium. To monitor CA9 shedding from short-term cultures of RCC explants, tumor fragments cut to 1 mm pieces (33 mg/mL) were rinsed with serum-free DMEM and cultured with or without IL-2 (100 ng/mL) in DMEM with 10% fetal bovine serum in 24-well plate and incubated at 37°C in a 5% CO_2 incubator for 3 days. To quantify CA9 expression, tumor fragments were evaluated by Western blot using anti-CA9 antibody.

Binding of Chaperone-Antigen Complex by Dendritic Cells

To FITC-label CA9 or BSA (control protein), FITC (Sigma) was added at 20 mol/L excess in 0.1 mol/L sodium bicarbonate/sodium carbonate. Free FITC was removed with a Sephadex G-25 column (Pharmacia). Proteins were subjected to SDS-PAGE to confirm FITC conjugation.

To assess binding to dendritic cells, FITC-conjugated proteins (10 μg/mL) were incubated for 20 min on ice with murine bone marrow-derived dendritic cells (see Generation of Immune Response with sCA9) at 1 × 10^6 cells/mL in 100 μL PBS containing 1% BSA. For the binding competition study, unlabeled CA9 or fucoidan was added at varying concentrations to 1 × 10^6 dendritic cells/mL at 4°C for 20 min. The dendritic cells were washed three times with 1% BSA/PBS and then incubated with 200 μg/mL FITC-CA9. The nucleus was counterstained with 4′,6-diamidino-2-phenylindole.

The cells were fixed with 1% paraformaldehyde (Fisher), examined by confocal microscopy (Bio-Rad), and analyzed by flow cytometry (Becton Dickinson).

Processing of Chaperone-Antigen Complex by Dendritic Cells

Dendritic cells were grown to 90% confluence and treated with 10 μmol/L MG132 or 10 mmol/L NH$_4$Cl for 2 h at 37°C. Untreated cells served as controls. Cells were cooled to 4°C for 30 min before adding CA9 at 10 μg/mL. The cells were kept at 4°C for an additional hour and then washed with cold RPMI 1640. The cells were then warmed to 37°C and harvested at 0, 0.5, 1, 2, 4, and 24 h time points, washed, and treated with radioimmunoprecipitation assay buffer (Sigma) for 15 min on ice to lyse cells. Lysate (20 μg) was subjected to Western blot analysis. The blots were probed with mouse anti-human CA9 antibody.

Short-term Kidney Tumor Culture from Surgical Specimens

Fresh human kidney tumors were obtained from the institutional tissue procurement service under an institutional review board-approved protocol (I53605). Tumor tissues were cut to 1 mm pieces, rinsed with serum-free RPMI 1640, suspended in DMEM with 10% fetal bovine serum, and incubated in 100 mm Petri dishes at 37°C in a 5% CO_2 incubator. To quantify CA9 shed from tumors, the culture medium was harvested from a suspension culture after 2 days. To quantify CA9 levels in the tumor, small tumor fragments were treated with radioimmunoprecipitation assay buffer. CA9 from the culture medium or cell extract was analyzed by Western blotting.

sCA9 was concentrated from RENCA-CA9 cells, which sheds a soluble form of CA9 that is 4 kDa smaller than CA9. RENCA-CA9 cells were cultured in 20 mL RPMI 1640 with 10% fetal bovine serum. Cells grown to 100% confluence were cultured for an additional 24 h in serum-free RPMI 1640. The culture medium was dialyzed for 24 h with PBS and spun at 4,000 rpm for 1 h in a concentrating tube. The concentration of sCA9 was measured by Western blot using purified CA9 as a standard. The medium from parental RENCA cells, which do not express CA9, was concentrated using the same protocol and served as a control.

Data Analysis

Error bars indicate SE for experiments done in triplicate. Differences in tumor growth were assessed using repeated-measures ANOVA. $P < 0.05$ was considered statistically significant. Statistical analysis was done using Stata 8.2 (StataCorp).

Results

CA9 as a Chaperone

HSPs are chaperones that complex intracellular proteins and facilitate proper folding and trafficking of newly synthesized proteins. At heat shock temperatures (43°C), HSPs protect cells by binding and preventing aggregation of intracellular proteins. The operational definition of HSPs includes three characteristics: (*a*) ability to bind client protein, (*b*) ability to prevent aggregation of client protein, and (*c*) ability to refold denatured protein.

To evaluate CA9 for chaperoning function, luciferase was used as a reporter protein (Fig. 1A). HSP110, which is a HSP with well-characterized chaperoning function (29), served as a positive control and ovalbumin served as a negative control. CA9, HSP110, or ovalbumin was mixed with luciferase at a 1:1 ratio. Immunoprecipitation was done with antibodies against CA9, HSP110, or ovalbumin and the complex was probed with antibodies to luciferase. HSP110 efficiently and irreversibility complexed luciferase at 43°C. CA9 was more efficient in complexing luciferase at room temperature than at 43°C.

To test whether formation of a complex between CA9 and luciferase protects luciferase from aggregation, CA9 and luciferase were mixed at 1:1 molar ratios and heated to 43°C (Fig. 1B). Protein aggregation was monitored over
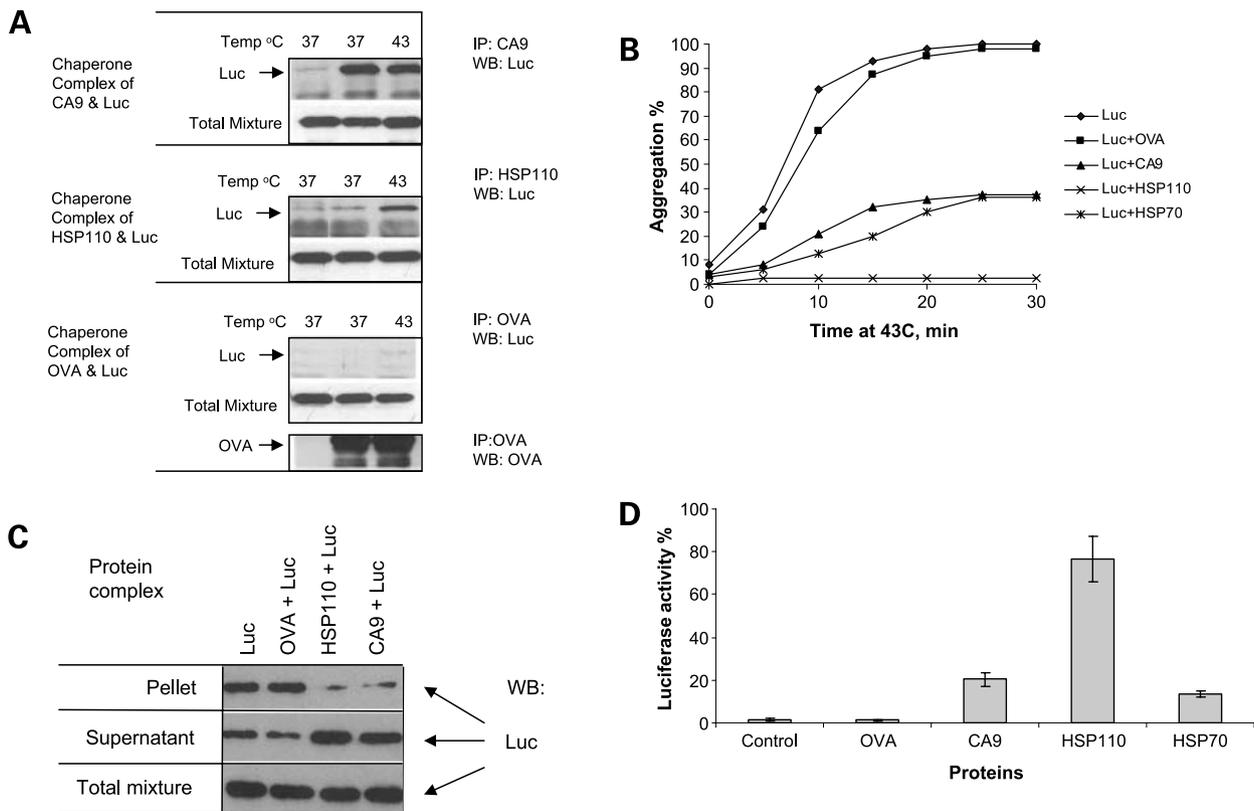
Figure 1. Chaperone-like function of CA9. An operational definition of a chaperone is a protein that can bind clients, prevent aggregation of clients at heat shock temperatures, and allow refolding of denatured proteins. CA9 has chaperone-like function. **A,** a pull-down assay was used to show that CA9 is capable of binding client proteins. Luciferase (*Luc*) served as the client protein, and CA9 formed a complex with luciferase at 37°C and 43°C. HSP110 served as a positive control and formed a complex with luciferase at 43°C but not at 37°C. Ovalbumin served as a negative control and had no chaperoning ability. Lanes with no chaperone (*lane 1*) were included as controls. Immunoprecipitation (*IP*) was done using antibodies against CA9, HSP110, or ovalbumin followed by Western blotting (*WB*) with anti-luciferase antibody. In the last row, Western blotting was done with anti-luciferase to confirm that ovalbumin pull-down was successful. **B,** HSP110 and HSP70 are classic chaperones and are able to bind and inhibit aggregation of client proteins at heat shock temperature. Recombinant CA9 was as effective as HSP70 in preventing aggregation of luciferase at 43°C. Ovalbumin served as a control protein. Luciferase aggregation was monitored over time by measuring absorbance at 320 nm. **C,** Western blot analysis confirmed that CA9 is able to prevent aggregation of luciferase and keep it in solution. Luciferase was heated to 43°C for 30 min with or without chaperone protein (CA9, HSP110, and ovalbumin). The total reaction mixture was centrifuged at $16,000 \times g$ for 15 min to separate the supernatant and pellet. In the control reactions (first two lanes), the majority of luciferase aggregated and was found in the pellet. CA9 and HSP110 were able to keep luciferase in solution at heat shock temperature. The total reaction mixture served as a loading control. **D,** classic HSPs chaperone denatured proteins and allow refolding. Luciferase was denatured at 43°C for 30 min in the presence of CA9, HSP110, HSP70, or ovalbumin (negative control). Rabbit reticulocyte lysate was added and refolding was assessed by monitoring the enzymatic activity of luciferase. The molar ratio of chaperone protein to luciferase was 20:1. Refolding ability correlated with ability to prevent aggregation of client proteins; CA9 and HSP70 were similar in its ability to prevent aggregation and refold luciferase. Mean ± SE of triplicate experiments.

time by optical densitometry. HSP70 is another well-characterized HSP. HSP110 and HSP70 were included as positive controls. HSP110 was able to completely prevent luciferase aggregation, and HSP70 and CA9 were equally effective in inhibiting luciferase aggregation. Ovalbumin was a negative control and had no ability to prevent luciferase aggregation. In a confirmatory experiment, CA9 was able to keep luciferase in solution at 43°C (Fig. 1C). A mixture of CA9 and luciferase was heated to 43°C and centrifuged. Both CA9 and HSP110 were effective in keeping the majority of luciferase in the supernatant and out of the pellet.

HSPs assist with folding of newly synthesized proteins and refolding of denatured proteins. To assay for this function, a heat-denatured enzyme can be combined with chaperones in rabbit reticulocyte lysate and restoration of enzymatic activity can be monitored. To assess the ability of CA9 to refold denatured protein, luciferase was used as the reporter enzyme (Fig. 1D). HSP110 was the most effective chaperone for allowing heat-denatured luciferase to be refolded. CA9 and HSP70 were equally effective. Ovalbumin served as a negative control and did not facilitate refolding.

CA9 Binding to Dendritic Cells

HSP are potent immunoadjuvants. Immunostimulation starts with HSP binding to dendritic cells in a receptor-specific manner. CA9 also bound dendritic cells in a saturable manner, indicating receptor-specific binding (Fig. 2A). CA9 binding was blocked by unlabeled CA9 (Fig. 2B) and fucoidan (Fig. 2C), which is a ligand for

scavenger receptors. Scavenger receptor A is one of many scavenger receptors on dendritic cells. CA9 binding was decreased when the binding assay was done using bone marrow-derived dendritic cells harvested from scavenger receptor A knockout mice (Fig. 2D).

Shed CA9 as a Chaperone

Previous reports describe a soluble form of CA9 shed from the surface of RCCs (25, 26). These reports were confirmed by blotting cell culture medium for CA9 (Fig. 3A). A soluble form of CA9 (sCA9), which was ~4 kDa smaller than the full-length CA9, was shed from a short-term culture of clear-cell renal tumors. However, normal kidney and papillary renal tumors did not shed CA9. sCA9 and CA9 were equally effective in preventing the aggregation of luciferase at 43°C (Fig. 3B).

Key binding studies were repeated using sCA9 with results identical to those obtained for CA9. sCA9 also bound dendritic cells in a saturable manner (Fig. 3C) and sCA9 binding was inhibited by unlabeled CA9 and fucoidan (Fig. 3D).

Delivery of Antigen to Dendritic Cells and CA9 Processing

Delivery of antigens to dendritic cells is an early step in generation of an adaptive immune response. Both CA9 and sCA9 were able to bind luciferase and deliver it to fresh murine dendritic cells (Fig. 4A). A complex of sCA9 and luciferase was added to dendritic cells at 4°C. Western blot analysis showed that luciferase bound to dendritic cells when complexed to sCA9 or CA9 but not when luciferase alone was added to dendritic cells. FITC-labeled CA9 bound to the surface of dendritic cells at 4°C as shown by confocal microscopy (Fig. 4B). When the cells were warmed to 37°C, labeled CA9 was internalized by dendritic cells.

After internalization of CA9, the next step in activation of an adaptive immune response is processing of CA9 by dendritic cells. Cell surface binding was monitored at 4°C. To monitor the status of internal CA9, cells were incubated at 37°C to allow intracellular processes to occur. Cell surface CA9 was washed and intracellular CA9 was measured by probing the cell lysate. At 37°C, intracellular
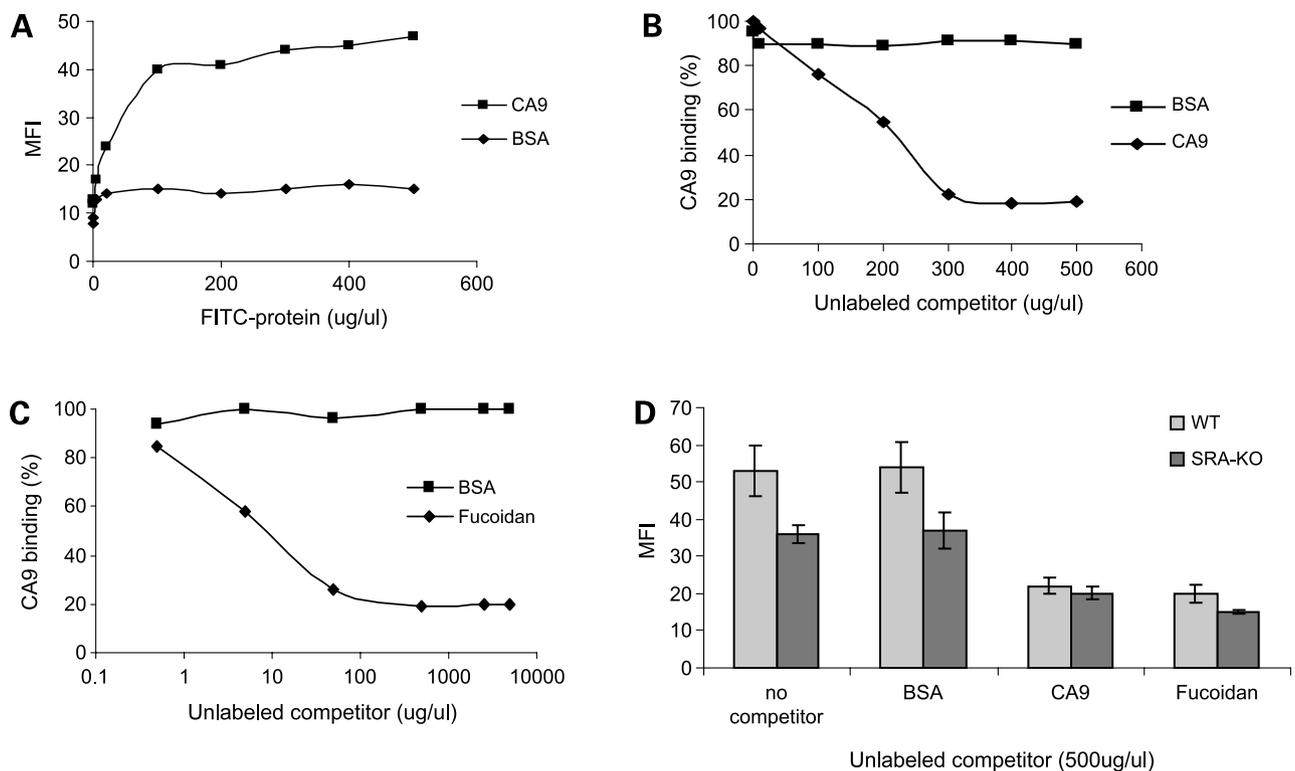
Figure 2. Binding of CA9 to dendritic cells. HSPs are chaperones with potent immunoadjuvant properties. **A,** classic HSPs are capable of binding dendritic cells and stimulating both innate and adaptive immune responses. CA9 bound dendritic cells in a saturable manner, suggesting receptor mediating binding. Bone marrow-derived dendritic cells (1 × 10⁶/mL) were incubated at 4°C with FITC-labeled CA9 or FITC-labeled BSA for 30 min and washed twice with 1% BSA/PBS. CA9 binding was assessed by measuring the mean fluorescence intensity using flow cytometry. **B,** binding of CA9 to dendritic cells was inhibited by unlabeled CA9. Bone marrow-derived dendritic cells (1 × 10⁶/mL) were incubated at 4°C with unlabeled CA9 or BSA at the indicated concentrations, washed, and then incubated with 200 μg/mL FITC-labeled CA9. **C,** binding of CA9 to dendritic cells was also inhibited by scavenger receptor ligand. Bone marrow-derived dendritic cells (1 × 10⁶/mL) were incubated at 4°C with fucoidan or BSA at the indicated concentrations, washed, and then incubated with 200 μg/mL FITC-labeled CA9. **D,** CA9 binding was decreased in dendritic cells from scavenger receptor A knockout mice when compared with dendritic cells from wild-type mice, suggesting that CA9 binding is in part mediated by scavenger receptor A. Bone marrow-derived dendritic cells (1 × 10⁶/mL) were incubated at 4°C with BSA, CA9, or fucoidan at 500 μg/mL before being washed and then incubated with 200 μg/mL FITC-labeled CA9. *Bars,* SE for experiments done in triplicate.
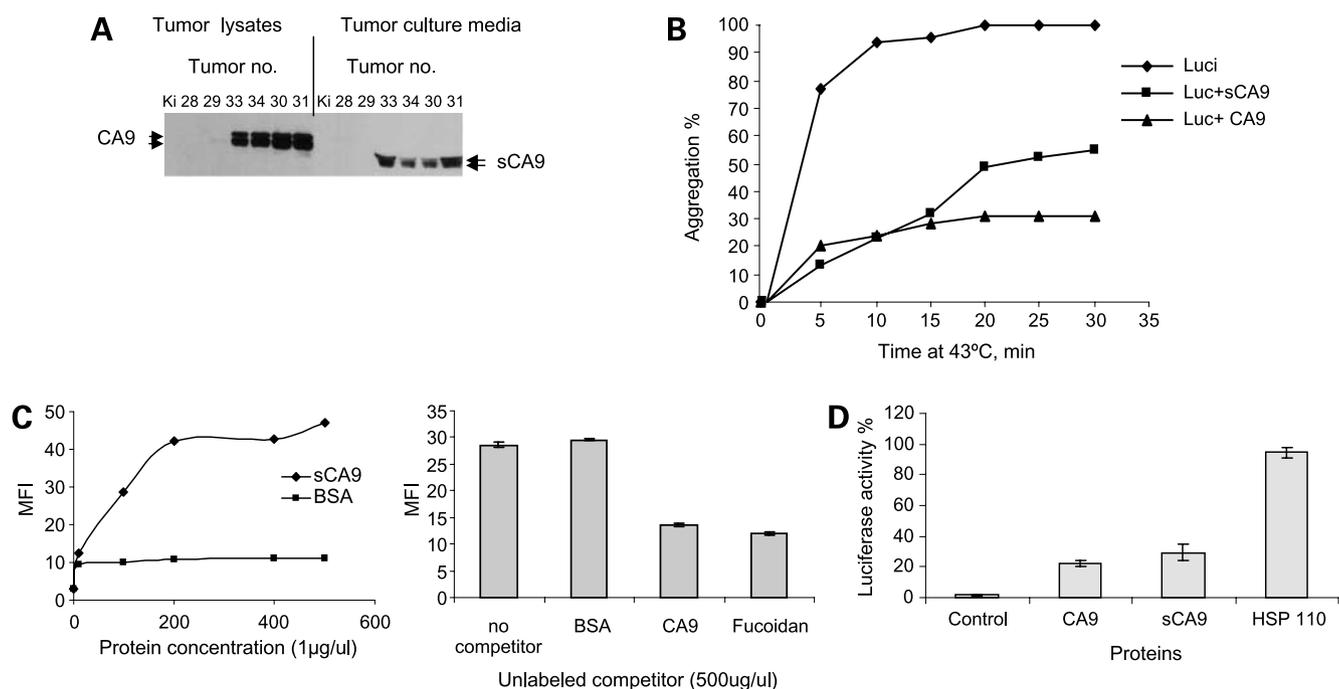
Figure 3. Soluble form of CA9 (sCA9). **A,** tumor cells shed sCA9. Cell lysates from human renal tumors were probed with anti-CA9 antibody, showing expression of full-length CA9. The same tumors grown in short-term cultures shed sCA9 that was ~4 kDa smaller in size than the full-length CA9. Clear-cell renal tumors (tumors 30-34) expressed and shed CA9. Normal kidney (*Ki*) and two papillary renal tumors (tumors 28 and 29) did not express or shed CA9. **B,** shed CA9 has chaperone-like properties. sCA9 was as effective as full-length CA9 in inhibiting the aggregation of luciferase at 43°C. Luciferase aggregation was monitored over time by measuring absorbance at 320 nm. **C,** sCA9 also bound dendritic cells in a saturable manner. Bone marrow-derived dendritic cells (1×10^6/mL) were incubated at 4°C with FITC-labeled sCA9 or FITC-labeled BSA for 30 min and washed twice with 1% BSA/PBS (*left*). As with CA9, sCA9 binding to dendritic cells was inhibited by unlabeled CA9 and fucoidan, suggesting that CA9 and sCA9 bind scavenger receptors on dendritic cells (*right*). Bone marrow-derived dendritic cells (1×10^6/mL) were incubated at 4°C with BSA, CA9, or fucoidan at 500 μg/mL before being washed and incubated with 200 μg/mL FITC-labeled CA9. *Bars,* SE for experiments done in triplicate. **D,** sCA9 allows refolding of denatured protein. Luciferase was denatured at 43°C for 30 min in the presence of CA9, sCA9, and HSP110. Rabbit reticulocyte lysate (*RRL*) was added, and refolding was assessed by monitoring the enzymatic activity of luciferase. The molar ratio of chaperone protein to luciferase was 20:1. CA9 and sCA9 were similar in its ability to refold luciferase. Mean ± SE of experiments done in triplicate.

CA9 rapidly increased but was nearly undetectable within 4 h (Fig. 4C). To evaluate the pathway for CA9 processing, intracellular CA9 levels were monitored in the presence of NH_4Cl and MG132, which inhibit lysosomes and proteosomes, respectively. Although both NH_4Cl and MG132 inhibited CA9 processing, MG132 was more effective in inhibiting CA9 processing. Therefore, intracellular CA9 is processed primarily by proteosomes, which process antigens for cross-presentation.

Stimulation of a Specific Antitumor Immune Response

To test whether CA9 can stimulate an antitumor immune response, a murine melanoma model was used to target a melanoma antigen, gp100. Recombinant CA9 and gp100 were complexed *in vitro* (CA9 + gp100) and used to immunize C57/BL6 mice. The mice were challenged with syngeneic B16 tumors stably transduced with gp100. Mice immunized with CA9 + gp100 had a significantly slower tumor growth (Fig. 5A) and longer survival when compared with any of the control groups ($P < 0.05$; data not shown). Immunized with CA9 + gp100 produced a gp100-specific IFN-γ response measured using the ELI-

SPOT assay (Fig. 5B) and a tumor-specific cytotoxic T-cell response measured using the ^{51}Cr release assay (Fig. 5C). Therefore, immune monitoring showed that CA9 is able to produce a gp100-specific cellular immune response.

Like full-length CA9, sCA9 was capable of stimulating a specific immune response. In the murine melanoma model described in Fig. 5A to C, human gp100 was used as the vaccine target. Therefore, gp100 itself produced a modest antitumor immune response; however, this model effectively shows that gp100 immunity is augmented by CA9. In a confirmatory study, a murine gp100 peptide was evaluated as a target for generating a specific cytotoxic T-cell response. Mice were immunized with dendritic cells treated with a complex of CA9 and peptide (CA9 + peptide) or sCA9 and peptide (sCA9 + peptide). Immunized mice developed peptide-specific CTL as determined using the ^{51}Cr release assay (Fig. 5D).

CA9 Expression and Shedding in Response to Cytokines

CA9 expression in the primary renal tumor has been reported to predict response to IL-2 therapy. As a screening study, CA9 expression was monitored in the human R6

RCC cell line after adding conditioned medium from WBCs treated with various cytokines (Fig. 6A). Conditioned medium was used because cytokines provide therapeutic benefit by stimulating immune cells rather than directly targeting tumors. CA9 expression increased in response to IL-2 and IFN-α but not IFN-γ.

Because CA9 has been reported to predict IL-2 treatment response, we asked whether short-term culture of RCC explants increases CA9 shedding in response to IL-2 (Fig. 6B). In all three clear-cell RCCs examined, CA9 shedding increased in response to IL-2. One papillary tumor with no baseline CA9 expression (tumor 29) shed low levels of CA9 after treatment with IL-2. IL-2 was applied directly to surgical specimens, which contain both tumor cells and immune cells.

Discussion

For patients diagnosed with RCC, accurately determining prognosis is useful for patient counseling, selecting treatment, and considering enrollment for clinical trials. CA9 is widely recognized as a biomarker for RCC as well as solid tumors in general; however, the mechanism linking CA9 to prognosis has never been established. We identify a novel function of CA9 that may provide an explanatory mechanism for the clinical observation in RCC.

Experimentally, CA9 has many of the functions attributed to HSPs. HSPs are ubiquitous molecules that function as intracellular chaperones, assisting with protein folding, complexing, and trafficking (30, 31). Although they are constitutively expressed, they are further induced by cellular stress such as heat, hypoxia, and glucose
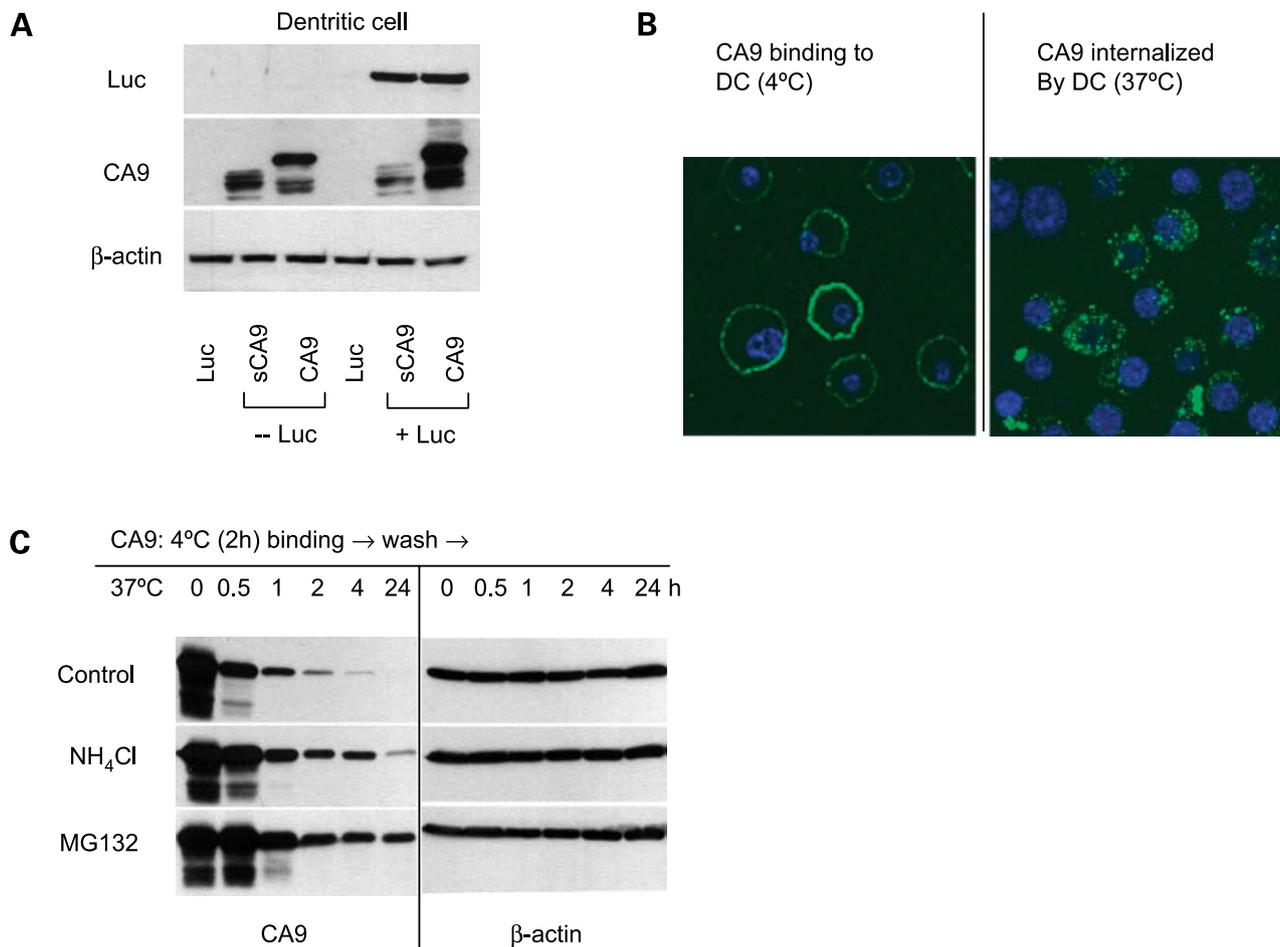
Figure 4. Delivery of client protein to dendritic cells and processing of CA9 by dendritic cells. **A,** both CA9 and sCA9 were able to bind and carry luciferase to dendritic cells. CA9 and sCA9 were complexed to luciferase at 37°C for 30 min and incubated with bone marrow-derived dendritic cells at 4°C for 2 h. CA9 and sCA9 without luciferase and luciferase alone were also incubated with dendritic cells and served as controls. Dendritic cells were washed twice to remove unbound CA9, lysed in radioimmunoprecipitation assay buffer, and probed by Western blot with anti-luciferase and anti-CA9 antibodies. **B,** CA9 was capable of binding dendritic cells and being internalized. Confocal microscopy showed FITC-labeled CA9 bound to the surface of bone marrow-derived dendritic cells at 4°C. Dendritic cells were washed to remove unbound CA9 and incubated at 37°C for 2 h, resulting in internalization of FITC-labeled CA9. The nucleus was counterstained with 4′,6-diamidino-2-phenylindole. **C,** dendritic cells were able to internalize and process CA9. CA9 was allowed to bind dendritic cells for 2 h at 4°C. Dendritic cells were washed twice and incubated in fresh medium at 37°C for the indicated time. Cell surface CA9 was washed and intracellular CA9 was monitored by Western blotting for CA9. β-Actin served as a loading control. In the untreated (control) group, CA9 processing resulted in decreasing intracellular CA9 detected by Western blotting. Pretreatment of dendritic cells with MG132 (proteosome inhibitor) or NH₄Cl (lysosome inhibitor) for 30 min at 37°C indicated that CA9 was processed by both pathways; however, the proteosomal pathway was dominant.
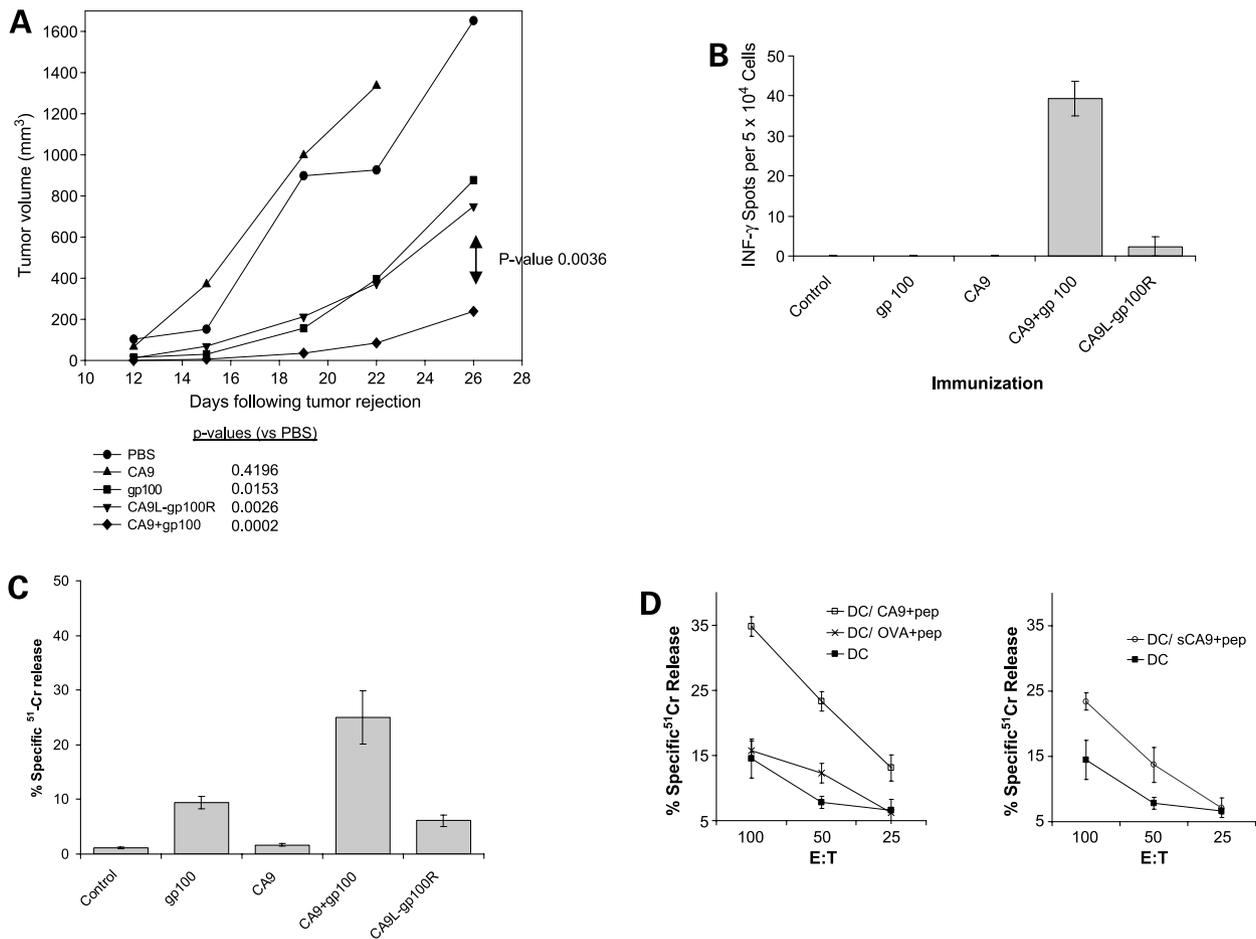
Figure 5. Immunization with CA9-gp100 complex in a murine melanoma model. **A,** immunization of C57/BL6 mice (5 per group) with CA9-gp100 complex (CA9 + gp100) produced an antitumor effect against B16-gp100 tumor cells. B16-gp100 tumor cells (2×10^5) were injected i.d. 7 d after three immunizations administered 7 d apart. Each line represents the mean tumor growth in 5 mice. *P* values based on repeated-measures ANOVA, which takes into account the variability within each group, are provided in the table comparing each group with the control mice injected with PBS. The rate of tumor growth was significantly different when comparing mice immunized with CA9 + gp100 and mice in any control group; for example, *P* = 0.0036 when comparing CA9 + gp100 group and mice immunized with injection of CA9 and gp100 into separate flanks (CA9L-gp100R). Similar results were obtained in three separate experiments. **B,** immunization with CA9 + gp100 elicited a gp100-specific IFN-γ response from CD8^{+} cells measured using the ELISPOT assay. Mean ± SE of experiments done in triplicate. **C,** immunization with CA9 + gp100 also elicited a tumor-specific cytotoxic T-cell response measured using the ^{51}Cr release assay. Mean ± SE of experiments done in triplicate. **D,** shed CA9 was capable of stimulating a specific cellular immune response against murine gp100 peptide (EGSRNQDWL). Immunization with dendritic cells treated with a complex of CA9 and gp100 peptide elicited a peptide-specific cytotoxic T-cell response measured using the ^{51}Cr release assay. C57/BL6 mice (3 per group) were immunized three times, 7 d apart, with 2×10^6 bone marrow-derived dendritic cells treated with the indicated protein-peptide complexes at 10 μg/mL and activated with lipopolysaccharide. Mean ± SE of experiments done in triplicate.

deprivation. During conditions of cell stress, HSPs promote cell survival by binding and protecting intracellular proteins (32).

The principal mammalian HSPs can be classified into sequence-related families that are also characterized by molecular size (33). HSP70 is one of the best characterized HSPs, and HSP110 is a member of the HSP70 superfamily. In this study, HSP110 was used as a positive control for chaperoning activity because previous studies have shown that HSP110 is one of the most effective chaperones for preventing the aggregation of client proteins and for stimulating a specific immune response that was dependent on both CD4^{+} and CD8^{+} T-cell populations (27, 34).

Experiments reported in the 1960s showed that tumor cells and lysates can protect mice against subsequent tumor challenges (35). Follow-up experiments using tumor fractions identified HSPs as the "active ingredient" providing immune protection (36). HSPs are promiscuously bound to a large repertoire of tumor antigens, which produces a tumor-specific immune response. It has been postulated that HSPs found outside a cell are recognized as a danger signal, indicating to the immune system the presence of damaged or diseased tissue (37).

Successful activation of the immune system against tumor is a complex process that ultimately results in an antitumor response. CA9 was able to generate an antitumor immune response in a murine melanoma model. C57/BL6

mice were vaccinated with a complex of CA9 and gp100 (CA9 + gp100) before being challenged with a syngeneic murine melanoma (B16) stably transduced to express gp100. Tumor growth was slowest and survival rates were highest in mice vaccinated with CA9 + gp100.

Human gp100 was used in the vaccine; therefore, it is not unexpected that gp100 itself produced a modest antitumor response. However, the tumor growth rate and survival in mice vaccinated with the CA9 + gp100 were statistically different when compared with all other vaccine groups. Furthermore, vaccination with CA9 + gp100 produced the most robust cellular immune response in mice as measured by ELISPOT and ^{51}Cr release assays. Therefore, CA9 augments the modest immunity seen with gp100 alone. Finally, in an all-murine model, vaccination with dendritic cells treated with a complex of CA9 and a murine gp100 peptide also produced a robust cellular immune response against gp100.

Our study suggests that CA9 and HSPs activate an immune response by a similar mechanism. For HSPs to serve as an immune adjuvant, it must form a complex with tumor antigens and bind antigen-presenting cells (38, 39). The ability of HSPs to form complexes with antigens is related to its chaperoning ability as measured with an aggregation assay. Cellular proteins must remain in solution to function; however, heat denaturation exposes hydrophobic regions and aggregates proteins. Like HSPs, CA9 was able to bind a client protein and protect it from heat-induced aggregation. Also, like HSPs, CA9 was able to bind and enable refolding of completely denatured protein, restoring protein function.

Receptors for HSPs have been identified on dendritic cells; for example, CD91 binds HSP90, HSP70, and calreticulin (40). HSPs have been shown to bind scavenger receptors (41). Our study suggests that CA9 binds to dendritic cells in a receptor-mediated fashion and scavenger receptors appear to play an important role in binding. Scavenger receptors are pattern recognition receptors, which promiscuously bind to a wide range of potential "danger signals." At least one of the scavenger receptors binding CA9 may be scavenger receptor A.

HSP complexed antigens can be processed and presented by antigen-presenting cells, resulting in antigen-specific, adaptive immunity (38, 42–45). Following CA9 binding to dendritic cell surface, CA9 was internalized and processed by dendritic cells primarily through the proteosomal pathway. The classic MHC I and MHC II pathways sample antigens from different sources. MHC I responses begin with proteosomal degradation of internally synthesized antigens. Classic MHC II responses begin with internalization of exogenous antigens and endosomal degradation. Proteosomal degradation of CA9 suggests that immune stimulation with CA9 leads to cross-presentation, which is the presentation of exogenous antigens on MHC I and activation of CD8$^+$ T lymphocytes. Cross-presentation is critical for a vaccine to be able to eradicate tumors.

Other investigators reported previously that a soluble form of CA9 is shed from the surface of renal and nonrenal tumors. Zavada et al. first reported that RCC cell lines and short-term cultures of patient tumors shed sCA9, which is 4 kDa smaller than the full-length CA9 (25). Shed CA9 was found in the serum and urine of renal carcinoma patients and was cleared from the blood after nephrectomy. CA9 shedding has been reported to be a regulated process that is metalloprotease dependent (26).

Our study confirmed that short-term cultures of RCC explants shed a smaller version of CA9, which likely corresponds to the extracellular domain of the full protein. To evaluate whether sCA9 has the same chaperoning ability as CA9, several of the assays reported for full-length CA9 were repeated with sCA9. Shed CA9 was able to function as a chaperone and prevent luciferase aggregation at heat shock temperatures. sCA9 complexed to luciferase was able to bind dendritic cells, effectively delivering luciferase for processing as an antigen. Finally, both CA9 and sCA9 can serve as an immune adjuvant capable of stimulating a specific CTL response against the abound antigen.
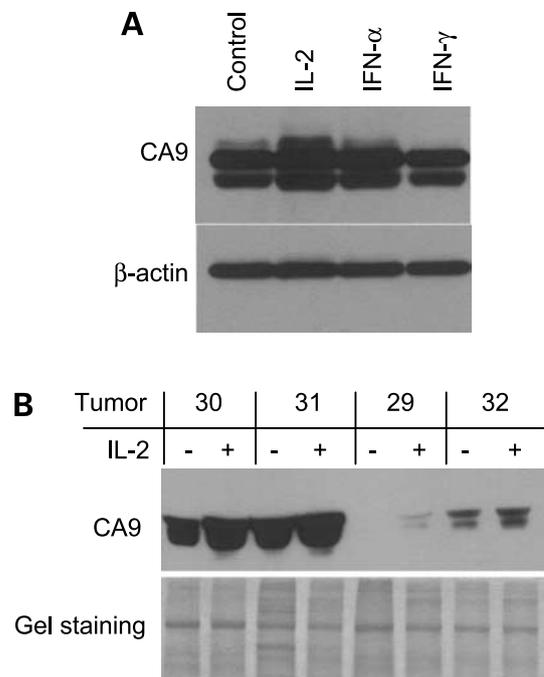
Figure 6. Effect of cytokines on CA9 shedding and expression. **A,** in patients with clear-cell renal carcinoma, increased CA9 expression has been associated with improved prognosis and response to immunotherapy. CA9 expression by the R6 RCC cell line was increased in response to medium conditioned with human WBCs treated with IL-2 and IFN-α. WBCs were separated from human blood and treated with IL-2, IFN-α, IFN-γ, or nothing (control) for 24 h to produce conditioned medium. R6 cells were treated with conditioned medium for 48 h before lysing and probing with anti-CA9 antibody. Conditioned medium was used because cytokines provide therapeutic benefit by stimulating immune cells rather than directly targeting tumors. **B,** CA9 shedding by primary tumor explants in short-term culture is increased in response to IL-2. Primary renal tumors were cultured with or without IL-2. The culture medium was probed with anti-CA9 antibody. The culture media were resolved by electrophoresis and stained with Coomassie blue to serve as a loading control. IL-2 was applied directly to surgical specimens, which contain both tumor cells and immune cells.

To the best of our knowledge, CA9 is the first reported example of a cell surface protein that can function as a chaperone with immunoadjuvant properties. Although structural domains responsible for this function have not been defined, this novel property of CA9 provides a mechanism for the observation that patients with high CA9-expressing RCCs have a better prognosis. CA9 expressed and shed by RCCs may contribute directly to producing a more robust antitumor immune response against RCC, which is an immunoresponsive tumor. In addition, CA9 shedding can increase in response to IL-2, providing a mechanism that contributes to IL-2 response. However, it is likely that other mechanisms contribute to clinical prognosis.

CA9 may play a more general role in biology. In cells with a normal hypoxia response, expression and shedding of CA9 has been shown to increase in response to hypoxia (26). Therefore, CA9 shedding provides a mechanism for recruiting the immune system in response to the harsh conditions of hypoxia. Under hypoxic conditions, sCA9 may sample the microenvironment and generate an immune response against extracellular antigens. HSPs such as HSP110 are induced by heat and efficiently complex antigens at heat shock temperatures. On the other hand, CA9 is induced by hypoxia and formed complexes with antigens at 37°C. This is consistent with our proposal that HSP and CA9 may have analogous roles in activating the immune system but under different conditions of stress.

Disclosure of Potential Conflicts of Interest

The authors have a provisional patent for the use of CA9 as an immunoadjuvant.

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