The Novel CA IX Inhibition Antibody chKM4927 Shows Anti-tumor Efficacy *In Vivo*

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Abstract. *Carbonic anhydrase IX (CA IX) is an attractive target for cancer therapy. Many anti-CA IX antibodies have been reported but few have been shown to possess inhibition activity. Furthermore, effective use of CA IX-inhibition antibodies for cancer immunotherapy has not been well-validated since data are mainly limited to in vitro assays. In this study, we established that chKM4927, an anti-CA IX chimeric antibody, recognizes CA IX and has CA IX-specific inhibition activity. ChKM4927 also retains antibody-dependent cellular cytotoxicity (ADCC) activity against CA IX-expressing cancer cells. Compared to controls, chKM4927 treatment (10 mg/kg) showed anti-tumor activity in the VMRC-RCW xenograft model in vivo. ChKM4927-attenuated ADCC activity showed equally effective anti-tumor activity. These results suggest that the CA IX-inhibition antibody chKM4927 has an anti-tumor effect in the VMRC-RCW xenograft model via an ADCC-independent mechanism.*

Regulation of pH is important for cancer cells to progress and migrate (1, 2). The tumor microenvironment causes high metabolic activity (3), which can lead to relatively acidic conditions in tumors compared to normal tissues (4, 5). Cancer cells protect themselves from the damage caused by dysregulated pH. Many proteins have been reported to import weak bases, such as HCO_3^-, and to export weak acids produced during metabolism, such as carbon dioxide, carbonic acid or lactic acid (6). Carbonic anhydrases (CA) are one of the major families of proteins involved in pH regulation and include cytosolic isozymes (CA I, CA II, CA III, CA VII and CA XIII), membrane-form isozymes (CA IV, CA IX, CA XII, CA XVI and CA XV), mitochondrial isozymes (CA VA and CA VB) and a secreted isozyme (CA VI). Among them, CA II, CA IX and CA XII are thought to be related to cancer progression through regulation of intra- and extracellular pH (7).

CA IX is a type I transmembrane protein with a molecular weight of 54-58 kDa located on the cell surface plasma membrane. CA IX contributes to intracellular alkalization by catalyzing the reaction $CO_2 + H_2O \rightarrow H^+ + HCO_3^-$ (8-10) and its expression is regulated by hypoxia inducible factor-1 (HIF-1). Normally, its expression is limited to gastrointestinal epithelial cells (11). However, it has been reported to be over-expressed in many types of cancers, such as non-small cell lung cancer, renal cell carcinoma, breast cancer and colon cancer (12). The expression of CA IX indicates a poor prognosis in breast and lung cancer (13, 14) and correlates with cell adhesion, migration and proliferation (15-17). Inhibition of its expression by siRNA leads to decreased migration, colony formation, drug resistance and tumor growth rates in xenograft models (18-21) suggesting that CA IX may be an attractive therapeutic target.

Although many CA inhibitors have recently been developed (22), only few studies have reported efficacy *in vivo* following CA IX inhibition (23-26). Furthermore, the structural similarities among the catalytic domains of the CA family proteins, especially between CA IX and CA XII (27, 28), make it difficult to create a CA IX-specific small-molecule inhibitor for evaluating the anti-tumor effects of

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CA IX-specific inhibition *in vivo*. Monoclonal antibodies are ideal for specifically recognizing CA IX. Though many anti-CA IX antibodies have been reported, few have been shown to possess inhibition activity (29, 30). Furthermore, the effective use of CA IX-inhibition antibodies for cancer immunotherapy has not been well-validated since the data are mainly limited to *in vitro* assays.

To evaluate the potential therapeutic effect of CA IX-inhibition antibody, we succeeded in creating chKM4927, a rat-human chimeric antibody that specifically recognizes CA IX. ChKM4927 showed inhibition activity against CA IX but did not influence other CA family proteins. We confirmed that chKM4927 has cytotoxic activity against human renal carcinoma cells (VMRC-RCW cells) *via* antibody-dependent cellular cytotoxicity (ADCC); however, the CA IX-inhibition activity did not affect the proliferation of these cells *in vitro*. Furthermore, we evaluated the anti-tumor activity of chKM4927 *in vivo*.

Materials and Methods

Animals. Sprague-Dawley (SD) rats and C.B.-17/Icr-scid Jcl (SCID) mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) and maintained under pathogen-free conditions. All animal experiments were performed in accordance with the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care International. This study and its protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Cell lines. The Chinese hamster ovary (CHO) cell line DG44 was kindly provided by Dr. Lawrence Chasin (Columbia University). The cancer cell lines HT29 (HTB-38), OSRC2 (RCB0735) and VMRC-RW (JCRB0813) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), RIKEN BioResource Center (Ibaraki, Japan) and the Japanese Collection of Research Bioresources (Osaka, Japan), respectively. The mouse myeloma cell line P3-U1 (CRL-1597) was purchased from ATCC.

Generation of transfected cells. Human CA IX (GenBank accession No. NM_001216.2) and domain-depleted CA *IX* (Figure 1A) cDNA were generated by polymerase chain reaction (PCR). Next, a Myc/His tag sequence (5'-GAACAAAAACTCATCTCAGAAGAGGATCTGA ATATGCATACCGGTCATCATCACCATCACCAT-3') was connected to the 3' end of the CA IX ORF by second-round PCR. The Myc/His-tagged cDNA (CA *IX*-Myc/His) was cloned into the pKANTEX93 vector and introduced into DG44 cells *via* electroporation to obtain G418-resistant clones.

Isolation of the anti-CA IX monoclonal antibody. Four-week-old female SD rats were immunized once with Bordetella pertussis adjuvant and CA IX-Fc, which consists of a CA IX extracellular domain (residues 41-406) linked to the human IgG1 Fc domain (including the hinge, CH2 and CH3 regions). They were then immunized three times with the adjuvant and CA IX-transfected CHO cells (CA IX/CHO). The spleens of these rats were removed three days after the final injection of the antigen and 2×10^8 splenocytes were fused with 2×10^7 P3-U1 cells in the presence of polyethylene glycol 1000 (Junsei, Tokyo, Japan). Cultured hybridoma cells were screened by flow cytometry of the supernatants using HT29 cells as target cells. After cloning twice by limited dilution, a stable clone was obtained (KM4927). The immunoglobulin class and subclass of KM4927 (IgG2a) were determined using anti-rat isotype-specific antibodies (Southern Biotech, Brimingham, AL, USA).

Production and purification of rat-human chimeric chKM4927. The heavy- and light-chain variable region cDNAs from the hybridoma cells producing KM4927 were isolated by PCR and cloned into the pKANTEX93 vector for production of the rat–human chimeric KM4927 antibody (chKM4927) (31). An Fc mutant of chKM4927 was generated in which the site for N-linked glycosylation in the Fc domain was eliminated by mutating the asparagine at position 297 to aspartic acid (chKM4927_N297D). The N297D mutation was introduced using PCR and mutagenesis was checked by sequencing. These vectors were then introduced into DG44 cells *via* electroporation and the transfected cells were grown in serum-free EX-CELL302 (Sigma-Aldrich, St. Louis, MO, USA). ChKM4927 and its modified antibody, chKM4927_N297D, were then purified from the supernatant of cultured cells using a Prosep-A column (Nihon Millipore, Tokyo, Japan).

Flow cytometry (FCM). To analyze chKM4927 binding to cell surface CA IX, CA IX-expressing cells were detached from culture plates using a 0.02% EDTA solution (Nacalai Tesque, Kyoto, Japan) and stained with 10 μg/ml of chKM4927 or control human IgG1 (Dako, Tokyo, Japan). To detect antibodies binding to the antigen, Alexa Fluor 488-labeled anti-human IgG1 (Invitrogen, Carlsbad, CA, USA) was added to the stained cells, which were subsequently analyzed by a FACS CANTO II instrument (BD Biosciences, San Jose, CA, USA) using the Flowjo ver. 7.6.5 software (Flowjo LLC, Ashland, OR, USA).

Analysis of the cross-reactivities of chKM4927 and CA family proteins by ELISA. Membrane-form or secreted CA family proteins (CA IV, VI, IX, XII and XIV) were plated onto a 96-well ELISA plate (Greiner, Esslingen, Germany) at a concentration of 1 μg/ml. After an overnight incubation at 4˚C, the plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20, blocked with 1% BSA in PBS and probed with 10 μg/ml chKM4927 for 1 h followed by an horseradish peroxidase (HRP)-conjugated anti-human IgG (Fc) antibody (American Qulex, San Clemente, CA, USA). ChKM4927 binding activity to CA proteins was visualized with 1 mM 2,2'-azino-di-[3-ethyl-benzothiazoline-6 sulfonic acid] diammonium salt (ABTS) and was measured at an absorbance of 405 nm.

Cell proliferation assay. VMRC-RCW cells (5×10^3) were seeded onto a 96-well plate and incubated in a CO_2 incubator overnight. Then, chKM4927 or isotype control was added at a final concentration of 5 μg/ml. After 72 h of incubation, Cell Titer-Glo (Promega, Madison, WI, USA) was used to count viable cells using a universal protocol.

Analysis of the binding of chKM4927 to FcγRIIIA using ELISA. The binding activity of chKM4927 and chKM4927_N297D to FcγRIIIA was measured by ELISA as described previously (32).

Measurement of the CA activity. A total of 15 μg of recombinant CA IX and 5 μg of chKM4927 were added to 20 ml of 0.02 mM Tris-HCl (pH 8.0) and stirred and cooled to 4˚C. Then, a pH electrode (Horiba, Kyoto, Japan) was inserted to monitor the

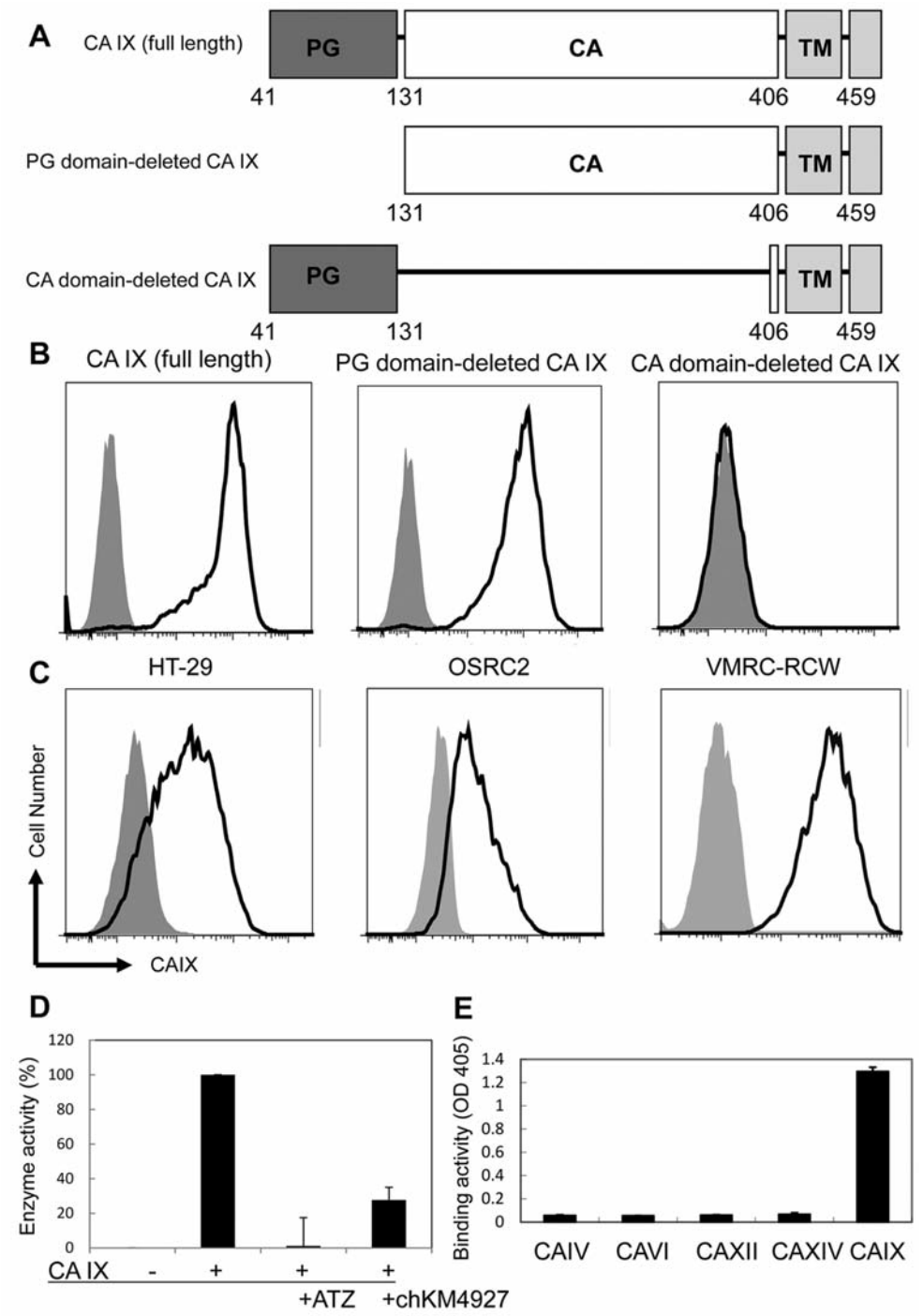

Figure 1. *ChKM4927 specifically recognizes the CA domain of CA IX and neutralizes CA IX activity. (A) The extracellular domain of CA IX is comprised of PG and CA domains. To determine the epitope of chKM4927, PG or CA domain-deleted CA IX constructs were created. (B, C) The binding of chKM4927 to transfectants expressing the modified CA IX described in (A) and to cancer cell lines was assessed by flow cytometry. The black line indicates the reactivity of chKM4927 at 10 μg/ml and the shaded histogram indicates the reactivity of a control antibody at 10 μg/ml. (B) The reactivities for CHO cells expressing human CA IX, human CA IX lacking the PG domain and human CA IX lacking the CA domain. (C) The reactivities for HT29, OSRC2 and VMRC-RCW cells. (D) The relative ability of acetazolamide (ATZ) and chKM4927 to inhibit the reaction, $CO_2 + H_2O \rightarrow H+ + HCO_3^-$, catalyzed by CA IX. Recombinant CA IX and chKM4927 or ATZ were added to 0.02 mM Tris-HCl (pH 8.0) and the solution's pH was monitored. The time required for the pH to change from 8.0 to 7.5 was recorded after CO_2-saturated water was added to the vessel. CA IX alone was considered as 100% enzyme activity. (E) ChKM4927 was added to a plate coated with recombinant protein (CA IV, CA VI, CA XII, CA XIV or CA IX). An anti-human Fc antibody was used to detect the chKM4927 bound to each recombinant protein.*

solution's pH. The time required for the pH to change from 8.0 to 7.5 was recorded after a 6–8 ml aliquot of CO_2-saturated water (4˚C) was added to the vessel. To determine the spontaneous CO_2 hydration rate, measurements were performed on protein-free "blank buffer" samples and on samples treated with recombinant CA IX protein. The inhibition rate of CA IX activity was determined as follows:

$$\text{CA IX activity (\%)} = (1-(T_1-T_2)/(T_1-T_3)) \times 100$$

where, T_1 is the time (in seconds) required for the pH to change from 8.0 to 7.5 in blank buffer, T_2 is the time spent in the buffer with recombinant CA IX protein and CA IX inhibitor and T3 is the time spent in the buffer with only recombinant CA IX protein.

Measurement of the ADCC and complement-dependent cytotoxicity (CDC) activities. ADCC and CDC assays were performed as described previously (32). Briefly, ADCC was detected by the lactate dehydrogenase (LDH) release assay using cryopreserved normal human peripheral blood mononuclear cells (PBMCs) (Precision Bioservices, Frederick, MD, USA) as effector cells and VMRC-RCW cells as target cells. CDC was detected by the cell proliferation reagent WST-1 (Roche, Basel, Switzerland) after incubation of cells with human sera complement (Sigma-Aldrich) and different concentrations of antibodies.

Mouse tumor model. VMRC-RCW (2.5×10^6) cells were injected subcutaneously into seven-week-old female SCID mice (CLEA Japan) one day after being pre-treated with PBS, 10 mg/kg of chKM4927 or chKM4927_N297D. After cell injection, 10 mg/kg of chKM4927, chKM4927_N297D or PBS were injected intraperitoneally twice a week for three weeks for a total of seven injections. The tumor volume was calculated using the following equation:

Tumor volume (mm_3)=0.5 × (major diameter) × (minor diameter)^2.

The *in vivo* results were statistically analyzed by a Kruskal-Wallis one-way analysis of variance and the Steel test and differences between the experimental groups were considered to be significant for values of $p<0.05$.

Results

Isolation of an anti-CA IX antibody with CA IX inhibition activity. To isolate anti-CA IX antibodies, SD rats were immunized with a CA IX-Fc fusion protein and CA IX/CHO. Hybridoma cells produced from spleen cells were selected based on their binding activities to a human colon cancer cell line, HT29. As a result, we obtained KM4927, a rat anti-CA IX antibody, which showed strong binding to CA IX/CHO and HT29. To evaluate the therapeutic potential of KM4927, a human-rat chimeric antibody, chKM4927, was produced. Its Fc region, human IgG1, was expected to have ADCC activity through human natural killer (NK) cells and human CDC activity.

The extracellular domain of CA IX consists of a carbonic anhydrase domain located close to the plasma membrane (CA, aa 135-391) and a proteoglycan domain at the N-terminal (PG, aa 53-111) (Figure 1A). To discover a chKM4927 binding domain, CA domain-deleted CA IX-transfected cells (DelCA/CHO) and PG domain-deleted CA IX-transfected cells (DelPG/CHO) were established. ChKM4927 bound to DelPG/CHO, but not to DelCA/CHO, suggesting that an epitope of chKM4927 is located in the CA domain (Figure 1B). ChKM4927 also could bind to CA IX/CHO, HT29, OSRC2 and VMRC-RCW cells (Figure 1C).

Next, we validated the effects of chKM4927 on the catalytic activity of CA IX by measuring the time required for the pH to change from 8.0 to 7.5 after adding CO_2-saturated water to a solution with or without chKM4927 in the presence of CA IX protein. Acetazolamide (ATZ) was used as a positive control, since it is known to be a pan-CA inhibitor. The pH of the solutions with chKM4927 or ATZ decreased more slowly than the solution containing only CA IX (Figure 1D).

Since there exist membrane-associated or secreted CA family proteins besides CA IX, we assessed the binding of chKM4927 to these proteins. As shown in Figure 1E, while chKM4927 recognized recombinant CA IX, it did not bind to recombinant CA IV, VI, XII or XIV. Taken together, these findings indicate that chKM4927 binds to some cancer cell lines and recognizes CA IX specifically among extracellular CA family proteins. Moreover, it can inhibit CA IX catalysis.

The effects of chKM4927 on CA IX-expressing cells in vitro. It has been reported that pH regulation by CA IX is related to cancer cell proliferation. To evaluate the inhibition activity of chKM4927 on CA IX-expressing cells *in vitro*, the proliferation rates of VMRC-RCW cells were determined in the presence of chKM4927. It was found that chKM4927 had no influence on the proliferation of VMRC-RCW *in vitro* (Figure 2A).

Since chKM4927 was expected to have ADCC activity through human NK cells and human CDC activity, we tested both ADCC and CDC activity in cell culture. Using VMRC-RCW cells as targets and PBMCs as effectors, we found that chKM4927 (10 μg/ml) showed ADCC activity that led to the death of more than 10% of all target cells (Figure 2B). The CDC activity of chKM4927 was not observed against VMRC-RCW cells (Figure 2C).

The effects of chKM4927 and chKM4927_N297D on a xenograft mouse model. There have been few reports regarding the *in vivo* effects of CA IX inhibition antibody. Thus, the potential of CA IX inhibition antibody for therapeutic use remains unclear. Our established antibody, chKM4927, can specifically recognize CA IX and has inhibition activity. Therefore, we evaluated the effects of chKM4927 *in vivo* in a xenograft mouse model using immunodeficient SCID mice. ChKM4927 is cytotoxic to VMRC-RCW *via* ADCC but not *via* CA IX inhibition or CDC *in vitro*. To assess the contribution of ADCC activity *in vivo*, we produced chKM4927_N297D, which has less ADCC activity, by replacing the asparagine with aspartic

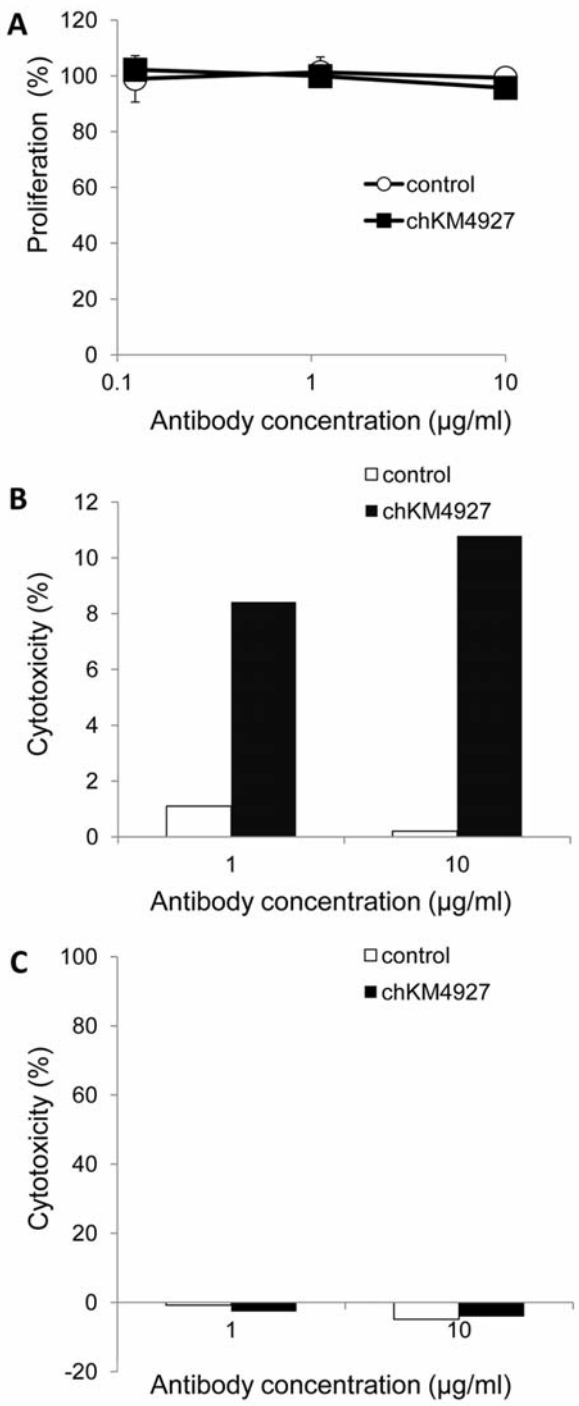

Figure 2. *In vitro anti-tumor activity of chKM4927. (A) VMRC-RCW cells were seeded and incubated overnight, followed by the addition of chKM4927 (black squares) or a control antibody (white circles). After 72 h, living cells were counted. (B) Effect on ADCC activity against VMRC-RCW cells in the presence of human PBMCs with chKM4927 (black columns) or a control antibody (white columns) as determined by 4-h LDH releasing assays. (C) Effect on CDC activity against VMRC-RCW cells in the presence of human complement with chKM4927 (black columns) or a control antibody (white columns) as determined by the WST-1 cell proliferation assay.*

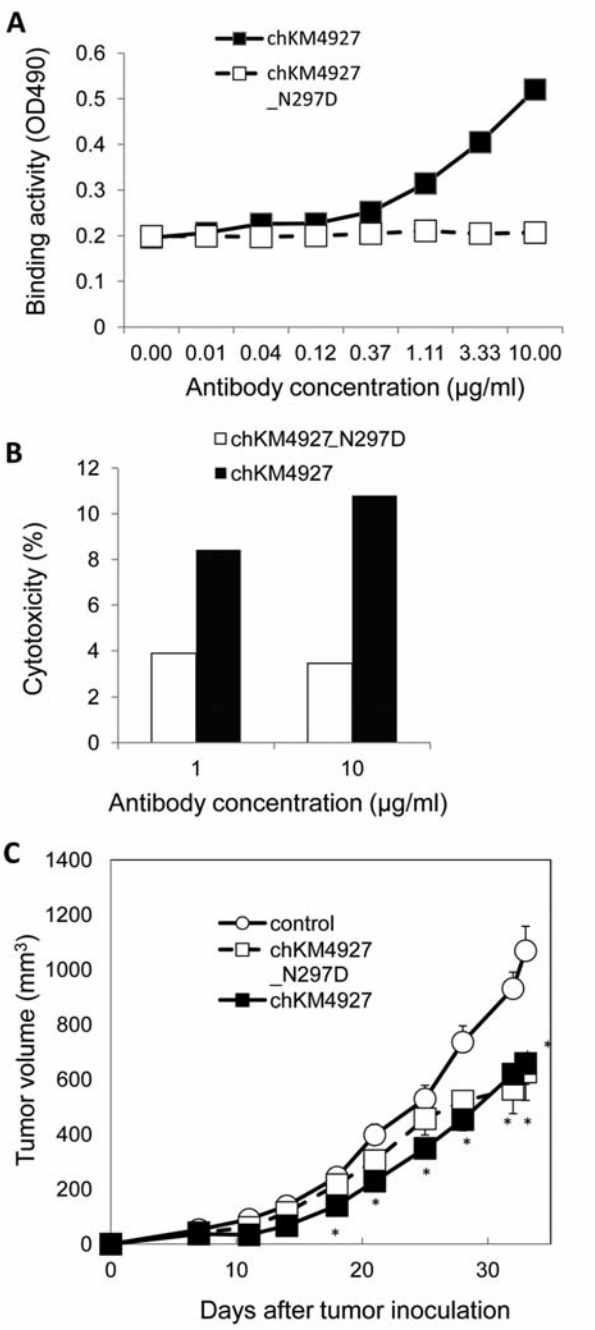

Figure 3. *In vivo anti-tumor activity of chKM4927 and de-glycosylated chKM4927 (chKM4927_N297D) in the VMRC-RCW xenograft mouse model. (A) The binding activities of chKM4927 (black squares) and chKM4927_N297D (white squares) to immobilized soluble FcγRIIIA were detected by peroxidase-labeled anti-human IgG1 antibody. (B) Effect on ADCC activity against VMRC-RCW in the presence of human PBMCs with chKM4927 (black columns) or chKM4927_N297D (white columns) as determined by 4-h LDH releasing assays. (C) The mean tumor volume±SE of VMRC-RCW tumors in SCID mice 32 days after cell injection and treatment with PBS (white circles), 10 mg/kg of chKM4927 (black squares) or 10 mg/kg of chKM4927_N297D (white squares) (intraperitoneally) (n=8-10) are indicated. The asterisks indicate significant differences vs. the PBS-treated group ($p<0.05$, Kruskal-Wallis and Steel test).*

acid at position 297 of the Fc domain. This domain is important for the binding of antibodies to the FcγRIIIA expressed in NK cells. Indeed, while chKM4927 showed dose-dependent binding to recombinant FcγRIIIA, chKM4927_N297D did not bind and caused less ADCC activity in VMRC-RCW cells (Figures 3A and 3B).

Since chKM4927_N297D caused reduced ADCC activity compared to chKM4927, it was injected to xenografted mice to assess the influence of CA IX inhibition alone on the xenograft tumor growth. VMRC-RCW cells were injected subcutaneously and xenograft-bearing mice were treated intraperitoneally with PBS or 10 mg/kg chKM4927 or chKM4927_N297D. At day 18 post-injection, chKM4927 had induced an anti-tumor effect compared with control. At day 32 post-injection, the anti-tumor effect of chKM4927_N297D was equal to that of chKM4927 suggesting that chKM4927 can inhibit the growth of VMRC-RCW xenograft tumors *in vivo via* an ADCC-independent mechanism.

Discussion

CA IX is over-expressed in various solid tumors and plays an important role in tumor cells by regulating the extracellular pH, especially in hypoxic regions that can cause severe damage to cells (12). Although many antibodies have been created since this molecule was discovered, no analyses of the effects of CA IX inhibition antibody *in vivo* have been reported. In the present study, we successfully created KM4927, a rat anti-CA IX antibody, which was, then, used to produce a rat-human chimeric antibody, chKM4927. ChKM4927 bound to several cancer cell lines and its binding was correlated with CA IX expression. The epitope of chKM4927 is likely in the CA domain since it could not bind to a CA domain-deleted CA IX transfectant (Figure 1B). Assessment of chKM4927 binding to secreted or membranous CA family proteins, such as CA IV, VI, XII and XIV, revealed that chKM4927 specifically recognizes CA IX (Figure 1E). Furthermore, chKM4927 showed inhibitory effects against the pH-lowering activity of CA IX (Figure 1D). This suggests that chKM4927 is a CA IX-specific inhibitor.

ChKM4927 showed ADCC activity against VMRC-RCW cells *in vitro* (Figure 2B). On the other hand, chKM4927 showed no CDC in VMRC-RCW cells (Figure 2C). We speculate that several factors, including the amount of CA IX and factors inhibiting complements, such as CD46, CD55 and CD59, expressed on the tumor cells could cause CDC activity against VMRC-RCW cells (33, 34). The CA IX-inhibition activity of chKM4927 had no effect on the proliferation of the cancer cell lines tested (Figure 2A). These results indicate that chKM4927 is cytotoxic to VMRC-RCW *via* ADCC but not *via* CA IX inhibition or CDC *in vitro*.

To evaluate the anti-tumor effects of chKM4927 *in vivo*, chKM4927 was injected into VMRC-RCW-xenografted mice. This resulted in reduced tumor size compared to the control group. We also administered chKM4927_N297D, which causes less ADCC activity through decreased FcγRIIIA binding (Figures 3A and 3B). Interestingly, chKM4927 and chKM4927_N297D both exerted anti-tumor effects *in vivo* and there were no significant differences between these antibodies after xenografting (Figure 3C). Further examination is necessary to elucidate the exact underlying mechanisms and *in vivo* efficacy of chKM4927. However, our results show that CA IX inhibition may be an important contributor to the tumor reduction in this model, while ADCC activity does not make a major contribution. This is contrary to the *in vitro* results but this discrepancy may have been caused by the tumor environment *in vivo*. The tumor growth could depend on CA IX differently *in vitro* than *in vivo*. Although CA IX has been reported to regulate the intra- and extracellular pH, resulting in protection of cells from damage caused under hypoxic conditions *in vivo*, a conventional 2D culture does not reflect the required condition of tumors (35). Further studies are needed to clarify the mechanism by which chKM4927 exerts its anti-tumor activities *in vivo via* the ADCC-independent mechanism.

CA XII is another membrane protein of the CA family that can complement CA IX functions (36). Although chKM4927 showed anti-tumor effects in a xenograft mouse model, the inhibition of CA IX may be insufficient to completely inhibit tumor growth because of functional compensation by CA XII. This consideration is supported by a report showing that decreased CA IX expression through siRNA leads to increased expression of CA XII in spheroid culture (19). Accordingly, it may be appropriate to create an inhibitor against both CA IX and CA XII. However, while CA IX is expressed in a limited number of healthy tissues, CA XII is more widely expressed. Therefore, its inhibition could cause excessive side effects. Indeed, a mutation in CA XII was recently reported to cause hyponatremia due to excessive salt loss *via* sweat (37). Thus, a pure CA IX mono-specific inhibitor is the best format for targeting CA family proteins to treat cancer while reducing major safety concerns.

Girentuximab is an anti-CA IX chimeric antibody with ADCC activity and no CA IX inhibition activity. Girentuximab demonstrated clinical benefit for kidney cancer patients in a phaseI/II trial (38). This result may suggest ADCC is a clinically important anti-tumor mechanism of CA IX antibodies. ^{124}I labeled-girentuximab has also been developed for diagnostic use against renal cell carcinoma and the phase III trial ended in 2010 with positive results (39). This proved that CA IX is reliably expressed in renal cell carcinoma and that girentuximab reaches the tumor region specifically. These results raise the possibility that an anti-

CA IX antibody with both CA IX-inhibition activity and ADCC activity, such as chKM4927, could show significant efficacy against renal cell carcinoma in a clinical setting.

To the best of our knowledge, this is the first report of the *in vivo* anti-tumor activity of a CA IX-inhibiting monoclonal antibody. Our results indicate that chKM4927's dual ADCC and CA IX-inhibition activity may have the potential for future clinical use.

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