The cysteine-rich core domain of REIC/Dkk-3 is critical for its effect on monocyte differentiation and tumor regression

RIE KINOSHITA[1,2], MASAMI WATANABE[3,4], PENG HUANG[2], SHUN-AI LI[2], MASAKIYO SAKAGUCHI[5], HIROMI KUMON[2,4] and JUNICHIRO FUTAMI[1]

[1]Department of Biotechnology, Division of Chemistry and Biochemistry, Graduate School of Natural Science and Technology, and [2]Innovation Center Okayama for Nanobio-Targeted Therapy, Okayama University; [3]Center for Innovative Clinical Medicine, Okayama University Hospital; Departments of [4]Urology, [5]Cell Biology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan

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Abstract. Reduced expression in immortalized cells (REIC)/Dickkopf (Dkk)-3 is a tumor-suppressor gene and has been studied as a promising therapeutic gene for cancer gene therapy. Intratumoral injection of an adenovirus vector carrying the human REIC/Dkk-3 gene (Ad-REIC) elicits cancer cell-specific apoptosis and anticancer immune responses. The cytokine-like effect of secretory REIC/Dkk-3 on the induction of dendritic cell (DC)-like cell differentiation from monocytes plays a role in systemic anticancer immunity. In the present study, we generated recombinant full-length and N-terminally truncated REIC/Dkk-3 to characterize the biological activity of the protein. During the purification procedure, we identified a 17 kDa cysteine-rich stable product (C17-REIC) showing limited degradation. Further analysis showed that the C17-REIC domain was sufficient for the induction of DC-like cell differentiation from monocytes. Concomitant with the differentiation of DCs, the REIC/Dkk-3 protein induced the phosphorylation of glycogen synthase kinase 3β (GSK-3β) and signal transducers and activators of transcription (STAT) at a level comparable to that of granulocyte/macrophage colony-stimulating factor. In a mouse model of subcutaneous renal adenocarcinoma, intraperitoneal injection of full-length and C17-REIC proteins exerted anticancer effects in parallel with the activation of immunocompetent cells such as DCs and cytotoxic T lymphocytes in peripheral blood. Taken together, our results indicate that the stable cysteine-rich core region of REIC/Dkk-3 is responsible for the induction of anticancer immune responses. Because REIC/Dkk-3 is a naturally circulating serum protein, the upregulation REIC/Dkk-3 protein expression could be a promising option for cancer therapy.

Correspondence to: Dr Junichiro Futami, Department of Biotechnology, Division of Chemistry and Biochemistry, Graduate School of Natural Science and Technology, Okayama University, 3-1-1 Tsushima-Naka, Okayama 700-8530, Japan
E-mail: futamij@okayama-u.ac.jp

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Introduction

Reduced expression in immortalized cells (REIC)/Dickkopf (Dkk)-3 gene is a member of the Dkk family, which consists of four members (Dkk-1 to -4). Dkk proteins regulate the canonical Wnt/β-catenin signaling pathway, which plays a critical role in cell proliferation and differentiation (1,2). Dkk-1, -2 and -4 interact with the low-density lipoprotein-related receptor 5 or 6 (LRP5/6) and affect Wnt/β-catenin signaling (3-5). REIC/Dkk-3 does not associate with LRP5/6, and its involvement in Wnt/β-catenin signaling remains controversial (6-8).

Unlike other Dkk family members, REIC/Dkk-3 is a tumor-suppressor gene whose expression is markedly reduced in various types of cancer cells and tissues (9-16). Overexpression of REIC/Dkk-3 with an adenovirus vector carrying the human REIC/Dkk-3 gene (Ad-REIC) induces endoplasmic reticulum (ER) stress-mediated apoptosis in cancer cells (17,18). We previously demonstrated that the N-terminal region of REIC/Dkk-3 is responsible for its cancer cell-specific induction of apoptotic activity (19). In addition, infection of normal cells with Ad-REIC resulted in the production of interleukin (IL)-7, which contributes to systemic anticancer immunoreactivity (17). Based on these findings, a phase I-IIa study of Ad-REIC gene therapy in prostate cancer patients is ongoing (20).

Recently, we found that secreted REIC/Dkk-3 protein induces differentiation from monocytes to dendritic cell (DC)-like cells (21). DCs control immune homeostasis by regulating both innate and adaptive immunity. Since DCs play a critical role in initiating cancer immunity, they have become an attractive target for cancer immune therapy. The mechanisms by which cytokines regulate DC development from hematopoietic stem cells have been extensively analyzed *in vitro* (22,23). For example, the addition of granulocyte/macrophage colony-stimulating factor (GM-CSF) and

IL-4 to the culture medium is a standard procedure to induce DC differentiation from monocytes, and it has been applied to the preparation of DC vaccines for cancer therapy (24,25). Differentiation of DC-like cells was observed when monocytes were treated with exogenous REIC/Dkk-3 protein at doses higher than 1 μg/ml (21); however, the naturally circulating REIC/Dkk-3 protein is found at 40-60 ng/ml in serum (26). To our knowledge, this activation is unique to the REIC/Dkk-3 protein among the Dkk family members, which has a relatively low (35-40%) sequence similarity in the Dkk family (1). In the present study, we analyzed the REIC/Dkk-3 protein to identify the region responsible for the induction of DC differentiation. In addition, the role of the REIC/Dkk-3 protein in immune activation was confirmed by examining its anticancer effects in response to intraperitoneal administration, and its effect on the activation of immunocompetent cells in blood.

Materials and methods

Construction of the expression plasmids. Recombinant REIC-Dkk-3 proteins were expressed using a previously developed supergene expression (SGE) system (27,28). The expression plasmid DNA [pIDT-SMART (C-TSC)-REIC] for expression of the full-length REIC/Dkk-3 (FL-REIC) protein was described previously (27). The cDNA fragment encoding an N-terminal truncated form of C-REIC [Arg142-Ile350] was amplified with PCR primers containing the *Eco*RI and *Bam*HI restriction sites. The PCR products were first cloned into the p3xFLAG-CMV-9 expression vector (Sigma-Aldrich, St. Louis, MO, USA) to express FLAG-tag fused C-REIC protein. To obtain efficient recombinant protein expression with the SGE system, the open reading frame was cloned into the pIDT-SMART (C-TSC) vector.

Preparation of the human REIC/Dkk-3 protein. Both FL-REIC and C-REIC were transiently expressed in FreeStyle™ 293-F cells (Life Technologies, Carlsbad, CA, USA) using Freestyle 293 Expression Medium and the 293 Fectin transfection reagent (Life Technologies), according to the manufacturer's instructions. Briefly, exponentially growing cells ($1x10^6$ cells/ml) with 180 ml media were prepared in a 500-ml flask. After transfection with 180 μg each of expression plasmid DNAs and 293 Fectin complex, the cells were cultivated using an orbital shaker (125 rpm) at 37˚C in the presence of 8% CO_2 for 4 days. Secreted proteins in the culture media were concentrated by Amicon Ultra centrifugal filter units (Millipore, Billerica, MA, USA), and the buffer was then replaced with 20 mM HEPES buffer (pH 7.2) using Sephadex G25M column chromatography (GE Healthcare, Piscataway, NJ, USA). Subsequently, the proteins were purified by anion exchange column chromatography (DEAE-Toyopearl 650M; Tosoh, Tokyo, Japan) and eluted with a linear NaCl gradient (0 to 0.7 M). The solution of the recombinant proteins was changed to PBS using a Sephadex G25M column, and then sterilized with 0.22 μm Millex-GV syringe filters (Millipore) and stored at -70˚C until use for the biological experiments.

Analysis of REIC/Dkk-3 degraded products. During the optimization of purification procedures for REIC/Dkk-3 proteins, degraded products were often detected on SDS-PAGE. This degradation converged to a 17-kDa band on SDS-PAGE, which was no longer degraded with long incubation times. This limited degradation product (C17-REIC) was analyzed for its amino-terminal sequence with a protein sequencer (Applied Biosystems 491), and carboxyl terminal amino acids were determined by amino acid analyzer (L-8500; Hitachi, Japan) after hydrazinolysis of the protein.

Preparation of the human monocytes. Human peripheral blood monocytes (PBMCs) were prepared from the blood of healthy donors by a standard method involving Ficoll-Paque centrifugation. The cell collection rate was determined by the trypan blue exclusion method. The survival rate was confirmed to be 99% or greater. For preparation of the monocytes, PBMCs were resuspended in LGM-3 (serum-free lymphocyte growth medium-3; Lonza, Walkersville, MD, USA). The cells adhering to a plastic dish (subjected to incubation in a 10-cm dish at 37˚C for 2 h) were used as monocytes. In some experiments, $CD14^+$ monocytes were separated using $CD14^+$ magnetic-activated cell sorting microbeads (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Purified $CD14^+$ monocytes were resuspended in LGM-3 medium.

Treatment of the human monocytes. $CD14^+$ monocytes were cultured in LGM-3 medium with or without DC differentiation factors. As a positive control, 2 ng/ml each of GM-CSF and IL-4 (both from R&D Systems, Minneapolis, MN, USA) were added to the medium. As for REIC/Dkk-3 proteins, 10 μg/ml of purified recombinant proteins was added. After cultivation for 7 days, the solution was stirred manually, and after 3 min, the number of DC-like cells per randomly selected visual field was counted with magnification of the slightly expanded photographs. The data were converted into a graph (n=5 visual fields). The cells were observed with a phase contrast microscope.

Western blotting. Purified $CD14^+$ monocytes were incubated for 6 h in LGM-3 medium with 2 ng/ml GM-CSF or 10 μg/ml REIC protein. Total cellular proteins were prepared from the treated cells, and western blot analysis was performed as previously described (21). Proteins were identified using the following antibodies: anti-phospho-Akt (Ser473), anti-phospho-glycogen synthase kinase 3β (GSK-3β) (Ser9), anti-GSK-3β, anti-phosphorylated signal transducers and activators of transcriptions (STAT)3 (Tyr705) and anti-phospho-STAT5 (Tyr694) (Cell Signaling Technology, Beverly, MA, USA).

Tumor-suppressive effects of FL-REIC and C17-REIC proteins in vivo. Murine renal carcinoma (RENCa) cells ($1x10^6$) were subcutaneously injected into mice (BALB/c, female, n=5). On days 3, 5, 7, 10, 12 and 14 after injection (provided that day 3 after injection was designated as the day of the start of administration of REIC proteins), 100 μg each of FL-REIC or C17-REIC, both proteins dissolved in 100 μl of PBS, or PBS as a control was intraperitoneally injected into mice. On day 17, the therapeutic effects were evaluated as tumor volume, and anticancer immune activity was measured before mice were euthanized. All experiments were conducted in accordance with the guidelines for animal experiments of our institution.

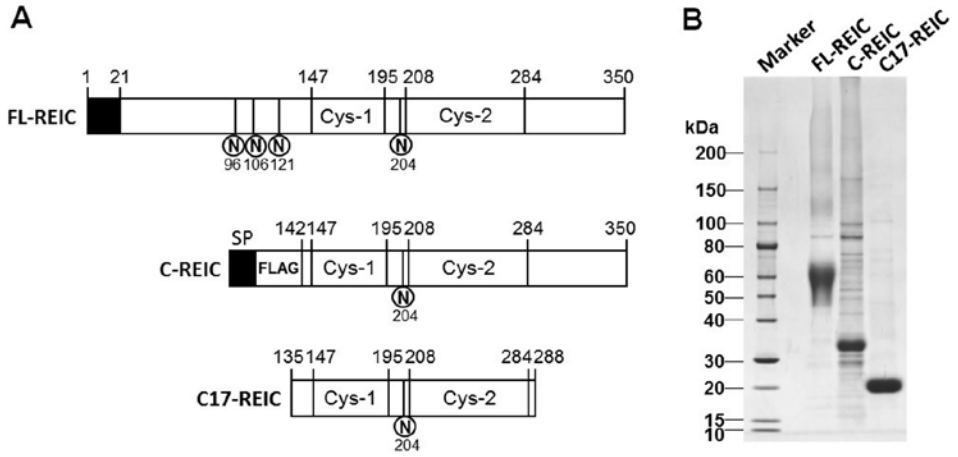

Figure 1. Purification of the human REIC/Dkk-3 proteins. (A) Schematic structure of human REIC/Dkk-3 proteins (FL-REIC, C-REIC and C17-REIC). The recombinant proteins possess one or four N-linked glycosylation sites (residues 96, 106, 121, 204) and an N-terminal signal peptide (black box: SP) and two cysteine-rich domains (Cys-1: residues 147-195 and Cys-2: residues 208-284). (B) The purity of recombinant human REIC/Dkk-3 proteins (FL-REIC, C-REIC and C17-REIC) was confirmed by SDS-PAGE stained with Coomassie brilliant blue (CBB).

Flow cytometry. EDTA (0.2% solution, 30 μl) was added to 750 μl of mouse blood collected from the inferior vena cava as an anticoagulant. Antibodies (1 μl each) with different fluorescent labels (purchased from eBioscience) were added to 30 μl of blood, stirred and incubated at 4˚C for 60 min to stain immunocompetent cells as follows: DCs (anti-CD11c antibody and anti-CD80 antibody) or cytotoxic T cells (anti-CD8 antibody and anti-CD69 antibody).

Subsequently, erythrocytes were lysed in a red blood cell lysis buffer. Cells were washed twice with PBS and resuspended in 200 μl of PBS to generate a solution for analysis. A total of $3x10^4$ cells were collected using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using CellQuest software (Becton Dickinson). An appropriate gate was set on the basis of the forward scatter pattern characteristic of these cells, and only cells within the gate were analyzed.

Statistical analysis. Data are expressed as the means ± standard error. Differences between two groups were analyzed using the unpaired Student's t-test, and $p<0.05$ was considered statistically significant.

Results

Production and purification of the REIC/Dkk-3 protein. To elucidate the molecular mechanism underlying the induction of anticancer immune responses by REIC/Dkk-3, the FL-REIC/Dkk-3 protein and the C terminal domain of REIC/Dkk-3 (C-REIC) containing two cysteine (Cys)-rich domains were produced in Freestyle 293-F cells (Life Technologies) (Fig. 1A). Secreted REIC/Dkk-3 protein was recovered from the culture medium of transfected 293-F cells on day 4. Approximately 100 mg of purified FL-REIC was obtained from a 1-liter culture using this system. Since the expression of C-REIC protein with the original signal peptide showed a low yield, the signal peptide was replaced by the Met-preprotrypsin leader sequence (PPT LS) preceding the FLAG coding sequence of the p3xFLAG-CMV-9 vector.

The stability of the FL-REIC protein was tested by incubation at 37˚C, which resulted in the detection of degraded products on SDS-PAGE. Although the degradation mechanism was unclear, this proteolytic degradation was enhanced with unpurified FL-REIC protein in a low-salt buffer. However, the degraded protein products converged in a band of ~17 kDa on SDS-PAGE, and longer incubation periods did not result in additional degradation products. Amino acid sequencing of this product resulted in the identification of Ser135 as the amino terminal residue and Phe288 as the carboxyl terminal residue (Fig. 1A). The purity of the REIC/Dkk-3 protein was determined as greater than 90% by SDS-PAGE (Fig. 1B).

The cysteine-rich domain of REIC/Dkk-3 is responsible for the induction of DC-like cell differentiation. Previous studies from our group showed that FL-REIC is a DC-like cell differentiation factor for monocytes when used at a range of 1-10 μg/ml (21). To investigate whether the C-REIC and C17-REIC proteins induce DC differentiation, $CD14^+$ monocytes were incubated with 10 μg/ml of purified REIC proteins. After a 7-day culture, imaging with a phase-contrast microscope showed DC-like differentiation of cells treated with GM-CSF/IL-4 and each of the three REIC proteins (FL-REIC, C-REIC and C17-REIC) (Fig. 2A). The number of DC-like cells per randomly-selected visual field (n=5) was counted, and the three types of REIC proteins had a comparable effect (Fig. 2B). These results indicated that C17-REIC, composed of two Cys-rich domains, is essential for the induction of DC-like cell differentiation.

REIC/Dkk-3-mediated phosphorylation of STAT3, STAT5 and GSK-3β plays a role in DC-like cell differentiation. Recently, intracellular activation of both STAT3 and STAT5 was found to play a role in the development of DCs (29-31). In our previous study, we showed that REIC/Dkk-3 phosphorylates STAT1 and STAT3 in monocytes (21). Furthermore, phosphorylation of GSK-3β on Ser9 by GM-CSF is thought to be involved in DC maturation (32). To evaluate the phosphorylation of STAT3, STAT5 and GSK-3β induced by REIC/Dkk-3,

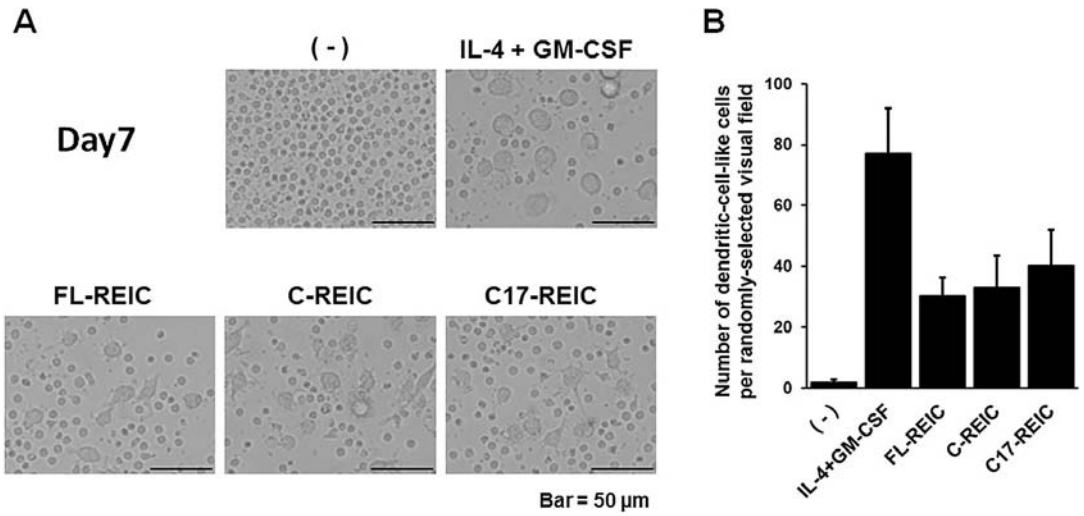

Figure 2. Induction of DC-like cell differentiation from monocytes by the REIC/Dkk3 proteins. (A) Phase contrast microscopic images of $CD14^+$ monocytes cultured alone (no addition), in the presence of GM-CSF and IL-4 (2 ng/ml each), or the FL-REIC, C-REIC and C17-REIC proteins (10 μg/ml) for 7 days. (B) The frequency of the occurrence of DC-like cells after treatment for 7 days was measured. The results are presented as the mean ± standard error (SE).

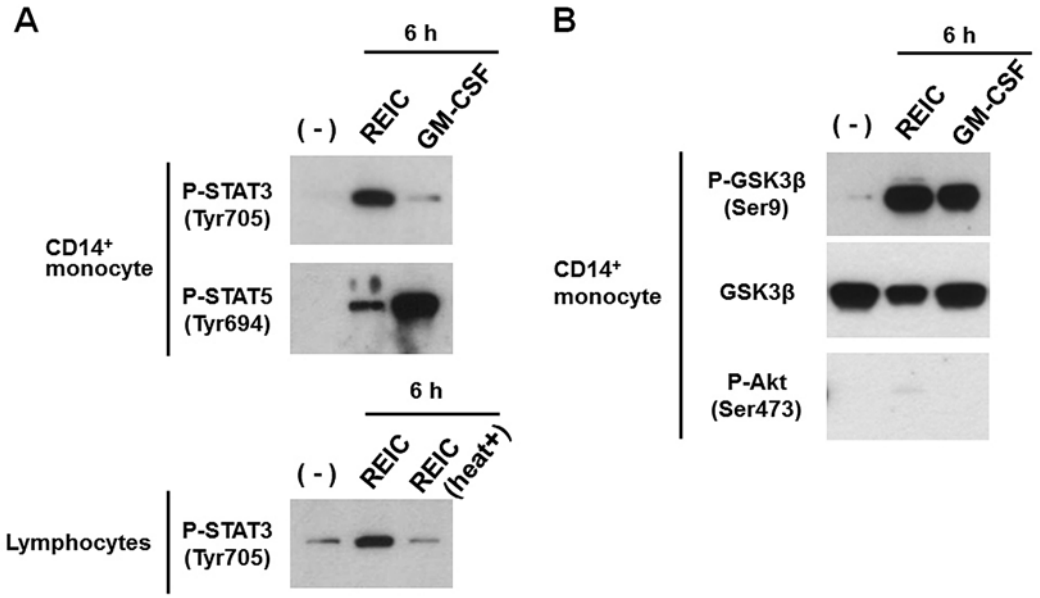

Figure 3. Lymphocytes and purified $CD14^+$ monocytes were treated with medium alone or 2 ng/ml GM-CSF or 10 μg/ml REIC protein for 6 h. Total cellular proteins were analyzed by western blot analysis. (A) Proteins were identified by the following antibodies: anti-phospho-STAT3 (Tyr705) and anti-phospho-STAT5 (Tyr694). (B) Proteins were identified by the following antibodies: anti-phospho-Akt (Ser473), anti-phospho-GSK-3β (Ser9) and anti-GSK-3β.

monocytes were treated for 6 h with REIC/Dkk-3 or GM-CSF. Both REIC/Dkk-3 and GM-CSF induced the phosphorylation of STAT3, STAT5 and GSK-3β in the monocytes, although the effective dose for REIC/Dkk-3 was much higher than that of GM-CSF (Fig. 3A and B). By contrast, heat treatment of REIC/Dkk-3 protein abrogated the effect on the phosphorylation of STAT3 in lymphocytes (Fig. 3A). Consequently, activation of STAT signaling and GSK-3β inactivation depending on Ser9 phosphorylation is a biological activity unique to the REIC/Dkk-3 protein, and it is not caused by possible contaminants, such as lipopolysaccharides. Since phosphorylated Akt also induces the phosphorylation of GSK-3β (33,34), we analyzed the activation of the PI3K/Akt pathway. Our results showed that Akt was not activated in response to REIC/Dkk-3 treatment (Fig. 3B). Taken together, these results revealed that REIC/Dkk-3 induces the phosphorylation of GSK-3β in monocytes independently from the PI3K/Akt pathway.

Intraperitoneal injection of REIC/Dkk-3 suppresses tumor growth via induction of cancer immunity. We previously demonstrated that intratumoral administration of FL-REIC inhibited tumor growth *in vivo* through the induction of cancer immunity (21). To investigate the antitumor potential of REIC/Dkk-3, FL-REIC and C17-REIC proteins were intraperitoneally injected into tumor-bearing mice (Fig. 4A). Significant tumor growth suppression was observed 17 days after the injection

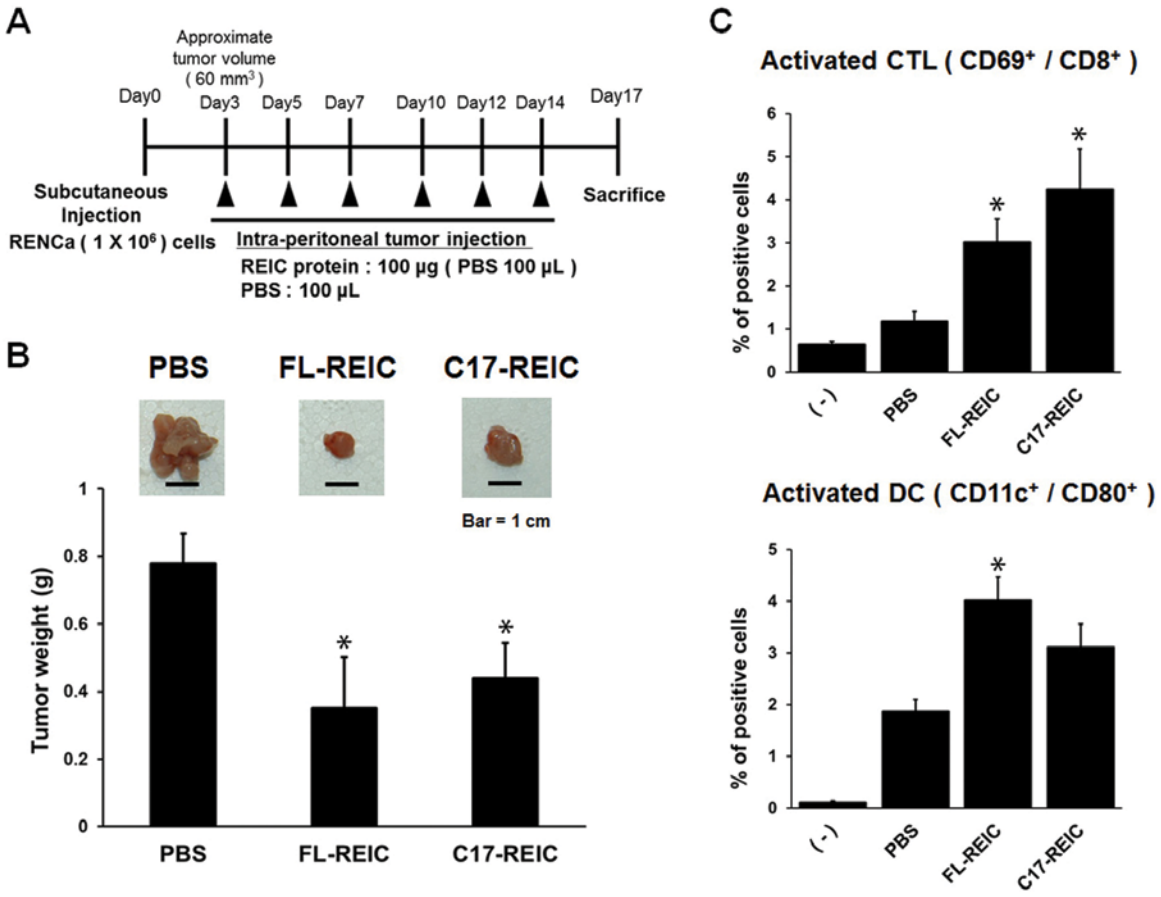

Figure 4. Tumor-suppressive effects of the REIC/Dkk3 proteins in the *in vivo* experiments. (A) Protocol illustrating the intraperitoneal administration of PBS, the FL-REIC protein and the C17-REIC protein. (B) Suppression of tumor growth (tumor weight) by intraperitoneal administration of the FL-REIC protein and the C17-REIC protein. The images show the inhibition of tumor growth in response to intraperitoneal protein administration. Scale bars, 1 cm. (C) The positive rate (%) of activated cytotoxic T lymphocytes (CTLs) ($CD69^+/CD8^+$) and activated DCs ($CD11c^+/CD80^+$) in each type of peripheral blood at the time (immediately before euthanasia) of completion of treatment with the REIC/Dkk-3 protein (FL-REIC or C17-REIC), in the untreated group, or in a group treated with PBS buffer. The results are presented as the mean ± standard error (SE). A significant difference ($^*p<0.05$) was observed between the REIC protein treatment group and the PBS treatment group.

of the REIC/Dkk-3 proteins. Tumor volumes were statistically significantly smaller in the group treated with both FL-REIC and C17-REIC than in the group treated with PBS (Fig. 4B). These antitumor effects of the REIC/Dkk-3 proteins were accompanied by *in vivo* induction of CTL ($CD69^+/CD8^+$) and DCs ($CD11c^+/CD80^+$) in the peripheral blood (Fig. 4C). Taken together, these results revealed that intraperitoneally injected REIC/Dkk-3 proteins exhibited antitumor effects mediated by the activation of systemic immunity, and the cysteine-rich core domain was essential for these biological responses.

Discussion

In the present study, we demonstrated the feasibility of anticancer protein therapy by using recombinant REIC proteins. High level production of recombinant REIC proteins was achieved by using Freestyle 293 cell suspension cultures and SGE high-level expression vector systems (27) with transient gene expression. During the process of FL-REIC protein purification, we identified a stable region designated as C17-REIC, composed of two Cys-rich domains. *In vitro* and *in vivo* assays using truncated forms of the REIC protein revealed that the Cys-rich core domain (C17-REIC) is critical for inducing cancer immunity, acting as a DC-like cell differentiation factor from monocytes. The N-terminal sequence of C17-REIC, SVGDEEGRRS, is the same sequence previously reported as the binding sequence for dynein light chain, Tctex1 (35). Although the detailed mechanism underlying the interaction between the secretory REIC/Dkk-3 protein and the intracellular Tctex1 protein remains unclear, the proteolytic processing of C17-REIC observed *in vitro* may reflect its intracellular biological action. Furthermore, we demonstrated that the C17-REIC protein acts as a tumor suppressor similar to the FL-REIC/Dkk-3 protein. Since the therapeutic effects of the protein depend on its structural integrity, it is important to minimize the risk of degradation, denaturation, aggregation, and precipitation, and storage conditions are important. Therefore, the fact that the robust C17-REIC domain is the domain responsible for protein function suggests that REIC possesses favorable features for protein-based therapy.

The results of the present study shed light on the molecular mechanisms underlying the induction of DC-like cell differentiation from monocytes by REIC/Dkk-3. The REIC/Dkk-3 protein induced the phosphorylation of GSK-3β at levels

comparable with the cytokine GM-CSF. Since GSK-3β phosphorylation is induced by various cytokines (33), the intracellular signaling pathway elicited in response to REIC/Dkk-3 stimulation may be shared with that of cytokines.

DC vaccine therapy is a promising option for cancer therapy. DCs exist in various populations characterized by different surface markers (23). In our previous study, we showed that the surface markers of DCs induced by REIC/Dkk-3 protein treatment were similar to those induced by GM-CSF and IL-4, except that the CD1a antigen was negative (21). Since REIC/Dkk-3 is ubiquitously expressed in normal tissues, whereas its expression is suppressed in many tumor tissues, REIC/Dkk-3 may play an important role in cancer immunity by regulating the differentiation of DCs. Indeed, intraperitoneal tumor injection of the REIC/Dkk-3 protein inhibited tumor growth and induced the activity of immunocompetent cells in blood in a mouse model of subcutaneous renal adenocarcinoma. The REIC/Dkk-3 gene is expressed in most human tissues (9) and the concentration of the secreted protein in normal human serum is 40-60 ng/ml (26), indicating that the risk of immunogenicity is low.

The findings of the present study support the hypothesis that the REIC/Dkk-3 protein is suitable for anticancer immunity medical treatment. REIC/Dkk-3 protein therapy holds promise as a method of immunotherapy. Ad-REIC gene therapy is a highly effective approach in various cancers, and has been shown to exert antitumor effects locally and systemically. In the future, REIC/Dkk-3 protein therapy may contribute to enhance the systemic antitumor effects of Ad-REIC therapy.

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