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

RIE KINOSHITA1,2, MASAMI WATANABE3,4, PENG HUANG2, SHUN-AI LI2, MASAKIYO SAKAGUCHI5, HIROMI KUMON2,4 and JUNICHIRO FUTAMI1

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

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 supgene 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 EcoRI and BamHI 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, 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 (1x10⁶ 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 resuspended in LGM-3 medium. After transfection with 180 µg each of expression plasmid DNAs and 293 Fectin complex, the cells were resuspended in LGM-3 medium with 2 ng/ml GM-CSF or 10 µg/ml REIC protein. 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).

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 (1x10⁴) 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.
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-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).
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.

\textit{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.
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 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.

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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