Abstract. The REIC/Dkk-3 gene has been reported to be a tumor suppressor and the expression is significantly down-regulated in a broad range of cancer cell types. The protein is secretory, but the physiological function remains unclear. This study demonstrated that recombinant REIC/Dkk-3 protein induced the differentiation of human CD14+ monocytes into a novel cell type (REIC/Dkk-3Mo). REIC/Dkk-3Mo resembles immature dendritic cells generated with IL-4 and GM-CSF. Both these cell populations exhibit similar proportions of CD11c+, CD40+, CD86+ and HLA-DR+ cells and endocytic capacity, but REIC/Dkk-3Mo is negative for CD1a antigen. An analysis of the signal transducers and activators of transcription (STAT) pathways revealed that REIC/Dkk-3 induces phosphorylation of STAT 1 and STAT 3. Furthermore, intratumoral administration of REIC/Dkk-3 protein significantly suppressed tumor growth with CD11c+ and CD8+ (dendritic and killer T cell marker, respectively) cell accumulation and enhanced anti-cancer cytolytic activity of splenocytes. These data indicated a cytokine-like role of REIC/Dkk-3 protein in monocyte differentiation that might be exploited therapeutically.

Introduction

The REIC (reduced expression in immortalized cells) gene was previously cloned and it is identical to the Dickkopf-3 (Dkk-3) gene, which is a member of the Dickkopf gene family (1). The expression of REIC/Dkk-3 is significantly down-regulated in a broad range of cancer cell lines and clinical tumors (1-6). The forced expression of REIC/Dkk-3, using a plasmid vector, inhibits cell growth in HeLa and liver cancer cell lines (3). An adenovirus vector carrying REIC/Dkk-3 selectively induces apoptosis in prostatic and testicular cancer cells but not in non-cancer cells, through the activation of c-Jun-NH2-kinase (JNK) and c-Jun (4,5). A recent study using malignant mesothelioma cells indicated that adenovirus mediated REIC/Dkk-3 overexpression triggers significant induction of apoptosis via endoplasmic reticulum (ER) stress (6). Ad-REIC treatment also inhibits the expression of Id-1, which works in cell cycle progression and anti-apoptosis (7). Therefore, accumulating evidence suggests that intracellular overexpression of REIC/Dkk-3 plays a distinct role in apoptotic induction and anti-oncogenesis. However, there have been few reports investigating the physiological functions of secretory or exogenous REIC/Dkk-3 protein. Although REIC/Dkk-3 is a member of the Dickkopf protein family known to interfere with Wnt/ß-catenin signaling via Wnt receptors (1,2,8), some studies have demonstrated that REIC/Dkk-3 does not affect the Wnt/ß-catenin signaling in these conditions (2,9). Until now, the molecular function and mechanism by which REIC/Dkk-3 protein exogenously acts on specific cell types remained unknown. This study presents novel evidence showing the immunological role of REIC/Dkk-3 protein in monocyte differentiation and in vivo tumor regression.

Materials and methods

Preparation of recombinant human REIC/Dkk-3 protein. Human REIC/Dkk-3 stable expression clones in CHO cells were established by electroporated transfection of the plasmids encoding the full length human REIC/Dkk-3 gene with a histidine tag and a puromycin-resistant gene. A total of 107 cells were cultured in 1 l of CHO protein-free medium (C5467, Sigma, St. Louis, MO) with 2 mM L-glutamine and 8 μM puromycin at 37˚C in 5% CO2 atmosphere for 1 week with gentle shaking. Using the TALON Resin (no. 635501, Clontech Laboratories, Mountain View, CA) in the culture supernatant, the histidine-tagged human REIC protein was collected and then subjected to Fast Protein Liquid Chromatography (MonoQ5/50GL column, GE Healthcare, Buckinghamshire, UK). The purified human REIC protein was dialyzed into...
phosphate-buffered saline (PBS) for stock and use and the stock solutions of the protein were maintained at -80°C until use.

**Preparation of human monocytes.** Human PBMC (peripheral blood mononuclear cell) were prepared from the blood of healthy donors by the standard procedure of Ficoll-Paque centrifugation. To purify the monocytes, the PBMC were resuspended in LGM-3 medium (Lonza, Walkersville, MD) and incubated in 10 cm plastic dish at 37°C for 2 h. After the incubation, the adherent cells were used for the monocytes. In some experiments, the CD14 positive monocytes were isolated from the PBMC using CD14+ magnetic activated cell sorting microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions.

**Culture conditions.** Purified monocytes were cultured either in LGM-3 medium alone or in the presence of recombinant human REIC/Dkk-3 protein (10 μg/ml, if not indicated). As a positive control for the differentiation of dendritic cells, the monocytes were cultured in LGM-3 medium supplemented with interleukin-4 (IL-4, 2 ng/ml, R&D Systems, Minneapolis, MN) and granulocyte-macrophage colony-stimulating factor (GM-CSF, 2 ng/ml, R&D Systems) (10,11). The cells were cultured in a final volume of 10-15 ml at 37°C in humidified incubators containing air with 5% CO2.

**Western blot analysis.** The total protein from treated cells or tumor tissue was extracted and Western blotting was done as described previously (4,12). Proteins were identified with the use of the following primary antibody at 1000x dilution; rabbit polyclonal anti-human REIC/Dkk-3 antibody raised in rabbit (no. 12-0809, R&D Systems, Minneapolis, MN), anti-phospho-STAT 3 (Try705) (no. 9131), anti-phospho-STAT 6 (Try641) (no. 9361).

**Flow cytometric analysis.** The floating and adherent cells were combined and approximately 5x10⁵ cells were incubated with the following PE-conjugated antibodies (5x diluted in 100 μl PBS) for 60 min on ice; CD11c (12-0116, eBioscience, San Diego, CA), CD14 (12-0149), CD1a (12-0019), CD80 (12-0809), CD83 (12-0839), CD86 (12-0869), HLA-DR (12-9956). Incubation with PE-conjugated immunoglobulin G (IgG) isotype-matched irrelevant antibodies (12-4714, 12-4732, eBioscience) were used as negative controls. After staining, 10⁶ cells were acquired on a FACSCalibur flow cytometer (Becton-Dickinson) and analyzed using the CellQuest software program (Becton-Dickinson). To examine the endocytic capacity of the cells, 50 μg of FITC-conjugated dextran (FITC-DX; FD40, Sigma, St. Louis, MO) was added to 0.5 ml of cell suspension (5x10⁶ cells/ml). The cells were then incubated at 4°C or 37°C for 1 h and analyzed by a flow cytometer.

**Animal experiments.** The RM9 mouse prostate cancer cell line was kindly provided by Dr T.C. Thompson (The University of Texas, Houston, TX) (13) and used in the animal experiments. To investigate the anti-tumor effects of REIC/Dkk-3 protein, 1x10⁶ RM9 cells were subcutaneously injected into the left thigh of C57BL/6 adult male mice with 0.1 ml PBS. On day 7, when the tumor volume reached ~300 mm³, each tumor was treated intratumorally with REIC protein or PBS alone as shown in Fig. 3A. In the other experiments shown in Fig. 4A, the orthotopic prostate cancer model with pre-established lung metastases was used (14,15) and the Ad-REIC or Ad-LacZ prepared as previously described (4) was injected into the orthotopic tumor on day 7. The injection of the agents was targeted to the center and periphery of each mass to deliver the agent diffusely and the tumor volume was determined as described previously (12).

**In vitro cytolytic assays.** To prepare the splenocytes, the spleen from each mouse was dissociated with glass slides and passed through a 70-μm cell strainer to obtain the cellular fraction. After lysing the red blood cells with ACK lysing buffer (Cambrex Bioscience, Walkersville, MD), the splenocytes were resuspended in 2% fetal bovine serum supplemented DMEM (Dulbecco’s modified Eagle’s medium, Invitrogen, Carlsbad, CA) and used for the cytolytic assays as an effector. RM9 cells were used for the target and the splenocytes were cultured with RM9 cells preseeded in a 96-well round bottom plate in duplicate in a ratio of effector/target of 100:1, 50:1, 25:1, 12.5:1. A total of 5x10⁵ target cells was applied per well. Supernatants were collected and lactate dehydrogenase released from lysed RM9 cells was measured using a non-radioactive cytotoxicity assay (Cytotox 96, Promega, Madison, WI). The percentage of lysed cells was calculated according to the following formula: (experimental release - effector spontaneous release - target spontaneous release)/(target maximal release - target spontaneous release) x 100%.

**Histological procedure.** Immunohistochemical staining was performed as previously described (12), using anti-CD11c and CD8 antibody (no. 550375 and no. 550281, respectively, BD Pharmingen, San Diego, CA) for the first antibody. To detect apoptotic cells in the tumor tissue, terminal deoxynucleotidyl transferase-mediated UTP end-labeling (TUNEL) assay was done using an In situ Cell Death Detection Kit, Fluorescein (Roche, Penzberg, Germany).

**Statistical analysis.** The data are shown as the mean ± SE. Unpaired Student's t-test was performed for the statistical analysis between the two groups and the difference was considered significant at p<0.05.

**Results**

REIC/Dkk-3 protein induces differentiation of monocytes into dendritic-cell like features. Full-length human REIC/Dkk-3 protein was produced in CHO cells and the purity was confirmed to be >95% by gel electrophoresis (Fig. 1A). Highly purified monocytes from PBMC were cultured in the presence of recombinant REIC/Dkk-3 protein (subsequently designated REIC/Dkk-3′/Mo) or with established protocols of dendritic cell differentiation by the addition of IL-4 and GM-CSF (subsequently designated IL-4+GM-CSF/D). During the first 2 days of incubation with REIC/Dkk-3 protein, approximately half of the REIC/Dkk-3′/Mo displayed cellular elongation (Fig. 1B). The change of cell morphology was
also observed in the IL-4+GM-CSFDC. The number of adherent REIC/Dkk-3Mo was markedly reduced after 5 days in culture and by day 7 most of the cells were suspended in the medium (Fig. 1B). The population of REIC/Dkk-3Mo indicates dendritic-cell features observed in IL-4+GM-CSFDC. The cell size of REIC/Dkk-3Mo is larger in comparison to the untreated monocytes on day 0. As for the monocytes treated in medium alone for 7 days, there were few cells with dendritic-cell like features and most of the monocytes appeared shrunken in comparison to the monocytes on day 0. To further verify the effects of REIC/Dkk-3 protein on the monocyte differentiation, human CD14+ monocytes were isolated from PBMC. In the CD14+ monocytes, the differentiation into the dendritic phenotype occurred dose-dependently after incubation with REIC/Dkk-3 protein (Fig. 1C). In the CD14 negative monocytes, no dendritic-like cells were observed after REIC/Dkk-3 incubation (data not shown).

The activation of STAT signaling is responsible for the differentiation of a variety of blood cells (16,17). Therefore, we analyzed whether REIC/Dkk-3 protein causes activation of STAT signaling by phosphorylation. The level of phosphorylation of STAT 3 was observed at 0.5 hr after the exposure. STAT 6, which is a signal transducer of IL-4, was not phosphorylated by treatment with REIC/Dkk-3 protein during the incubation period.

**Phenotypic characterization of the monocytes cultured with REIC/Dkk-3 protein.** The influence of REIC/Dkk-3 protein on the differentiation of monocytes into the dendritic cell phenotype was then verified (10,11). The REIC/Dkk-3Mo on day 7 was analyzed for specific surface markers by flow cytometry and IL-4+GM-CSFDC was used as the positive control for immature dendritic cells (Fig. 2B). In the monocytes cultured with medium alone for 7 days, propidium iodide (PI) staining in flow cytometry showed <5% of the cells were viable (data not shown). The proportion of CD11c+ was similar between the REIC/Dkk-3Mo and IL-4+GM-CSFDC populations. A lower proportion of CD40+ and CD80+ cells were observed in REIC/Dkk-3Mo in comparison to IL-4+GM-CSFDC. On the other hand, the proportion of CD86+, HLA-DR+ and CD14+ cells in REIC/Dkk-3Mo was significantly higher in comparison to IL-4+GM-CSFDC. The CD1a surface antigen was positive in the IL-4+GM-CSFDC, but not detected in REIC/Dkk-3Mo. CD83, which is a marker for mature dendritic cells, was negative in both cell populations. The endocytic capacity of these cell types...
was further evaluated by measuring the uptake of FITC-labeled dextran particles. After 7 days of culture with REIC/Dkk-3 protein or IL-4+GM-CSF, the cells were exposed to FITC-DX for 1 h at either 4°C (a background control for non-specific binding) or 37°C (active uptake). The internalization of FITC-DX by REIC/Dkk-3Mo was equivalent to that of IL-4+GM-CSFDC.

Intratumoral administration of REIC/Dkk-3 protein suppressed tumor growth in RM9 subcutaneous tumor model. The elevated expression of dendritic-cell markers with dendritic cell-like features indicated that the population of REIC/Dkk-3Mo might possess immunological capacities such as antigen presentation (10,11). Since REIC/Dkk-3 is a tumor suppressor gene and significantly down-regulated in a broad range of cancer cells (1-6), the gene product should also have anticancer functions. In order to investigate the anti-cancer effects of REIC/Dkk-3 protein, the in vivo experiment shown in Fig. 3A was performed. A significant suppression of tumor growth was observed on day 12 and 14 in the REIC/Dkk-3 treated group (Fig. 3B). A histopathological analysis with hematoxylin and eosin staining revealed massive degeneration of the tissues in the REIC/Dkk-3 injected tumors, but not in those treated with PBS (Fig. 3C). These findings were consistent with the tumor growth inhibition observed after the REIC/Dkk-3 treatment. Higher magnification in the injected sites revealed that the infiltration of many lymphocytes was observed within the area of the necrotic cancer cells with less hematoxylin-stained nuclei. Since the in vivo immunological activity of REIC/Dkk-3 protein was conceivable in terms of the differentiation of the dendritic cell-like phenotype, an immunohistochemical analysis of CD11c+ was performed in the tumor tissue specimens. A highly significant accumulation of CD11c positive cells was observed on day 21 in REIC/Dkk-3Mo.
Dkk-3 treated subcutaneous tumors but not in the controls (Fig. 3D). In addition, the infiltration of CD8 positive killer T cells was also detected in the serial sections. In vitro cytolytic assays revealed the enhanced cytotoxic activity of splenocytes from REIC/Dkk-3 protein treated mice against the RM9 prostate cancer cells (Fig. 3E).

Overexpression of REIC/Dkk-3 protein in RM9 orthotopic prostate tumor suppressed the lung tumor formation in pre-established metastasis model. Adenovirus mediated REIC/Dkk-3 overexpression was previously demonstrated to induce significant apoptosis in injected tumor sites and robust anti-tumor effects (4-6,15). In vitro REIC/Dkk-3 overexpression by Ad-REIC in cancer cells resulted in plenty of REIC/Dkk-3 protein secretion into the culture media (data not shown). Taken together, these findings lead to the hypothesis that the anti-cancer immune up-regulation by Ad-REIC treatment in primary tumor sites could trigger anti-tumor effects even at the distant tumor site. To test this hypothesis, an orthotopic RM9 prostate tumor model with pre-established lung metastasis was used (14) for the experiments as shown in Fig. 4A. The elevated expression of intratumoral REIC/Dkk-3 protein was first verified by Western blotting (Fig. 4B). The apoptotic induction by intratumoral Ad-REIC treatment was also confirmed by TUNEL staining (Fig. 4C). Consistently, the growth of orthotopic prostate tumors was strikingly inhibited 14 days after Ad-REIC injection in comparison to the controls (Fig. 4D). Moreover, the number of pre-established lung tumors significantly decreased in the Ad-REIC treated group (Fig. 4E). In vitro cytolytic assays revealed the enhanced anti-cancer immune activity of splenocytes from Ad-REIC treated mice against the RM9 prostate cancer cells (Fig. 4F).

Discussion

We currently elucidated the specific function of REIC/Dkk-3 protein in monocyte differentiation to a dendritic-cell phenotype. This is the first study to clearly demonstrate the exogenous action of REIC/Dkk-3 protein. The morphological features of REIC/Dkk-3Mo and the expression pattern of dendritic-cell markers on the surface are similar to those of IL-4+GM-CSFDC. These cell types also share the capacity of endocytosis, demonstrated by their FITC-DX uptake. However, the REIC/Dkk-3Mo and IL-4+GM-CSFDC phenotypes were distinctly different in terms of the expression of CD1a and CD14 surface markers. In addition, the expression pattern of
activated STAT families tends to differ between these cell types. Further studies are therefore necessary to determine the location of REIC/Dkk-3Mo in the monocyte differentiation pathway. It is also understood that the differentiation of monocytes is intricately regulated by the interleukin and interferon cytokine family (10,11). Furthermore, a specific cytokine treatment induces the activation of STAT family protein to induce the cellular differentiations (16,17). Therefore, exogenous REIC/Dkk-3 protein might possess a cytokine-like function through the activation of STAT 1 and STAT 3 during the REIC/Dkk-3Mo differentiation.

This study further demonstrated the in vivo anti-tumor effects of REIC/Dkk-3 protein to be accompanied by the up-regulation of systemic immunity against RM9 cancer cells. These findings are consistent with the significant cell death and infiltration of immunological CD11c+ and CD8+ cells observed in the treated tumor tissues. There was a prominent accumulation of CD11c positive cells, thus indicating the
possibility that REIC/Dkk-3 protein may also differentiate mouse monocytes into the CD11c+ dendritic-like phenotype observed under in vitro conditions. It is also conceivable that the subsequent cross-talk between the CD11c+ cells and CD8+ T lymphocytes might stimulate anti-cancer immunity. With regard to the cytotoxic effects of the REIC/Dkk-3 protein itself, in vitro preliminary experiments were performed to examine the direct effects in RM9 cells. Even at the concentration of 20 μg/ml, there were no significant cytotoxic changes associated with REIC/Dkk-3 treatment. Therefore, the in vivo anti-tumor effect of REIC/Dkk-3 protein largely depends on the induction of enhanced systemic anti-cancer immunity. When the orthotopic RM9 prostate tumor was treated with Ad-REIC, a significant tumor reduction was observed with local apoptotic induction. The systemic immunity against RM9 cancer cells was enhanced in the Ad-REIC treated group, thus suggesting the intratumoral overexpression of REIC/Dkk-3 protein and the local apoptotic cell death to possibly trigger the immunological modulation. Moreover, the distant anti-tumor effects in lung metastatic lesions were observed as well as at the primarily injected tumor site. Taken together, the in vitro monocyte differentiation by REIC/Dkk-3 protein into the dendritic phenotype and the in vivo results strikingly indicate that either exogenous or secreted REIC/Dkk-3 protein at the tumor site might activate the immune cells and thereby enhance the anti-cancer immunity.

The findings described herein shed new light on the functions of REIC/Dkk-3 protein in connection with both monocyte differentiation and anti-cancer immune processes. In addition, the differentiated monocytes such as REIC(Dkk)-3Mo, appear to be involved in the anti-cancer immunity. Therefore, REIC/Dkk-3 is an attractive immunological target which may help in the development of novel therapeutic strategies against human malignancies.

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