Adenovirus-Mediated Overexpression of REIC/Dkk-3 Selectively Induces Apoptosis in Human Prostate Cancer Cells through Activation of c-Jun-NH$_2$-Kinase

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Abstract
Alteration in genes which takes place during malignant conversion and progression could be potential targets for gene therapy. We previously identified REIC/Dkk-3 as a gene whose expression is reduced in many human cancers. Here, we showed that expression of REIC/Dkk-3 was consistently reduced in human prostate cancer tissues in a stage-dependent manner. Forced expression of REIC/Dkk-3 induced apoptosis in human prostate cancer cell lines lacking endogenous REIC/Dkk-3 expression but not in REIC/Dkk-3-proficient normal prostate epithelial and stromal cells. The apoptosis involved c-Jun-NH$_2$-kinase activation, mitochondrial translocation of Bax, and reduction of Bcl-2. A single injection of an adenovirus vector carrying REIC/Dkk-3 showed a dramatic antitumor effect on a xenotransplanted human prostate cancer. Thus, REIC/Dkk-3 could be a novel target for gene-based therapy of prostate cancer. (Cancer Res 2005; 65(21): 9617-22)

Introduction
Selective elimination of tumor cells is a key issue in treating human cancer. During malignant conversion and progression, various genetic changes take place in cells that could be potential targets of cancer gene therapy. Among the genes that exert a selective killing effect on cancer cells when overexpressed are p53 (1, 2) and mda-7/interleukin-24 (mda-7/IL-24; ref. 3). The Dickkopf (Dkk) gene family is known to interfere with Wnt signaling via Wnt-receptors (4, 5). Wnt genes play pleiotropic roles in critical biological contexts including development, cell growth/differentiation, and cancer (6). Although Dkk family members (four genes are known thus far in humans) are only vaguely understood, they probably fulfill important functions as well. We previously identified REIC/Dkk-3 as a gene whose expression is reduced in many human cancers (7–10). Forced expression of REIC/Dkk-3 using a plasmid vector inhibited expression of REIC/Dkk-3 selectively induced apoptotic cell death in human prostate cancer cells via activation of c-Jun-NH$_2$-kinase (JNK) and exerted a marked curing effect on xenotransplanted human prostate cancer.

Materials and Methods
Reagents, cells, and culture. Normal human prostate epithelial cells (PrEC) and prostate stromal cells (PrSC) cells were purchased from Cambrex (Baltimore, MD) and cultivated using medium recommended by the supplier. The prostate cancer cell lines PC3, DU145, and LNCaP were provided by American Type Culture Collection (Rockville, MD). Normal human fibroblasts (OUMS-24) were established by one of the authors (11). HAM’S F-12 K medium, RPMI 1640, and DMEM (Nissui, Tokyo, Japan) were used for PC3, DU145 and LNCaP, and OUMS-24, respectively, with a supplement of 10% fetal bovine serum. Subfractionation of cells was done using a Mitochondrial Fractionation Kit (Active Motif, Carlsbad, CA). A JNK inhibitor, SP600125, and a membrane-permeable Bax inhibitor, V5, were purchased from Biomol (Plymouth Meeting, PA) and Sigma Genosys (Woodlands, TX), respectively.

Human prostate tissues. LandMark low-density prostate tissue microarray (Ambion, Austin, TX) was used for immunostaining of REIC/Dkk-3. Fresh prostate biopsy samples were obtained from 40 patients under conditions permitted by the Ethical Board of our Graduate School. Twenty samples each had Gleason scores of ≤7 and scores of 8 to 10, respectively. Immunologic analyses. After fixation with 4% paraformaldehyde, cells and tissue sections were immunostained with anti-human REIC/Dkk-3 antibody raised in our laboratory, followed by treatment with Alexa fluor R594-conjugated goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR). Bcl-2 antibody was used for Bax and Bcl-2, rabbit anti-human Bax antibody (Upstate Cell Signaling Solutions, Lake Placid, NY) and mouse anti-human Bcl-2 antibody (BD Biosciences, San Diego, CA) were used, respectively. Vectashield mounting medium with 4′,6-diamidino-2-phenylindole or with propidium iodide (Vector Laboratories, Burlingame, CA) was used for counterstaining of cell nuclei. SyBR Green I (Cambrex) was used for nuclear staining in tissue sections. Signal intensity of the stained samples was quantitated using the computer software Scion Image Beta (Scion, Frederick, ML). Western blot analysis was done as previously described. The antibodies used were as follows: rabbit anti-human REIC/Dkk-3 antibodies raised in our laboratory for REIC/Dkk-3; Apoptosis Sampler I kit (BD Biosciences) for Bcl-2 and Bcl-xL; rabbit anti-human Bax antibody (Upstate Cell Signaling Solutions); mouse anti-human tubulin antibody (Sigma, St. Louis, MO); mouse anti-horse cytchrome c antibody (Upstate Biotechnology, Lake Placid, NY); mouse anti-mouse mitochondrial Hsp70 (Abcam, Cambridge, United Kingdom); rabbit anti-human c-Jun antibody, rabbit anti-human phospho-c-Jun (Ser63) antibody, rabbit anti-human stress-activated protein kinase/JNK antibody, and rabbit anti-human phospho-stress-activated protein kinase/JNK (Thr183/Tyr185) antibody (Cell Signaling Technology, Beverly, MA); mouse anti-β-galactosidase antibody (Calbiochem, San Diego, CA).

Real-time quantitative reverse transcription-PCR. Real-time PCR was done under the conditions recommended by the manufacturer using a LightCycler rapid thermal cycler instrument (Roche Diagnostic, Meylan, France). The primers used for real-time PCR were as follows:

Note: Supplementary data for this article are available at Cancer Research Online.
REIC/DKK-3 (forward) 5'-GTAAGTCCCTCCCTGGCTTG-3', REIC/Dkk-3 (reverse) 5'-AAGCCAGACTGTAAGCCTC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward) 5'-GGGTGTTGAAACCATGAGAAGTATGA-3', GAPDH (reverse) 5'-TGCTAACAGTTGGTTGCTGC-3'. The products were checked by a melting point analysis, electrophoretic mobility, and direct sequencing. Standard curves for calculation of the number of transcripts were made using plasmid containing the respective inserts. The results are shown as molar ratios of REIC/Dkk-3 to GAPDH transcripts.

Adenovirus vector carrying REIC/Dkk-3. For overexpression of REIC/Dkk-3, a full-length cDNA was integrated into a cosmid vector pAXCAwt and transferred into an adenovirus vector by the COS-TPC method (Takara Bio, Shiga, Japan). An adenovirus vector carrying LacZ gene was used for monitoring infection efficiency.

Apoptosis assay. Detection of DNA ladders was done under conventional conditions. Briefly, DNA was gently extracted after lysing cells with Triton X-100, treated with RNase and proteinase K, and electrophoresed on 2% agarose gel. Detection of DNA ladders was done under conventional conditions. Briefly, DNA was gently extracted after lysing cells with Triton X-100, treated with RNase and proteinase K, and electrophoresed on 2% agarose gel.

In vivo experiments. PC3 cells (2.5 × 10^6 in 50 µL PBS) were mixed with 50 µL Matrigel (BD Biosciences) and s.c. injected into the right flank of 8-week-old BALB/C nude mice (SLC, Hamatsu, Japan). One week after injection, when the tumor diameter reached ~5 mm, 2.5 × 10^6 plaque-forming units of an adenovirus vector carrying full-length REIC/Dkk-3 cDNA (Ad-REIC) or LacZ (Ad-LacZ) in a 100 µL buffer were injected intratumorally. The same volume of buffer was injected as a negative control. The size of tumors was measured every 3 days over 30 days after the injection. The volume was calculated using an empirical formula, \( V = \frac{1}{2} \times [(\text{the shortest diameter})^2 \times (\text{the longest diameter})] \). The experiments were done according to a guideline determined in our university.

Results and Discussion

Reduction of REIC/Dkk-3 expression in human prostate cancer cells and tissues. We first examined its expression in various cell lines. In normal human fibroblasts (OUMS-24), and prostate epithelial (PrEC) and stromal (PrSC) cells, REIC/Dkk-3 protein was detected as two major bands of ~60 and ~68 kDa in size (Fig. 1A). REIC/Dkk-3 gave rise to several bands of different apparent molecular size, probably due to variable glycosylation levels (10). REIC/Dkk-3 protein was barely detected in three prostate cancer cell lines and four cancer cell lines of other origin. Lack of expression of REIC/Dkk-3 protein in the prostate cancer cell lines was confirmed by immunostaining (Fig. 1B). Quantitative RT-PCR also revealed a reduction in REIC/Dkk-3 mRNA levels in the prostate cancer cell lines determined by real-time quantitative RT-PCR and expressed as molar ratios to those of GAPDH. *, \( P < 0.05 \) compared with OUMS-24. D, immunohistochemistry for REIC/Dkk-3 in normal and benign prostate hyperplasia (BPH) tissues and prostate cancer tissues at Gleason stages 4 and 9. Insets, low-magnification tissue staining with SyBR Green. E, quantitative image analysis for REIC/Dkk-3 protein in LandMark tissue microarray (left, 6 cases each) and in freshly isolated human prostate cancer tissues (right, 20 cases each). The intensity of signals was quantitated using the computer software, Scion Image Beta. BPH, benign hypertrophic prostate tissues; G, Gleason stages; *, \( P < 0.05 \); **, \( P < 0.01 \).
These results extend those that we have obtained previously by Northern blot analysis (7).

**Selective induction of apoptosis in human prostate cancer cell lines by overexpression of REIC/Dkk-3.** To examine the possible use of REIC/Dkk-3 as a tool for targeted gene-based therapy, we overexpressed REIC/Dkk-3 using a replication-deficient adenovirus vector. The REIC/Dkk-3 protein level in PC3 cells infected with the vector carrying REIC/Dkk-3 (Ad-REIC) at a multiplicity of infection (MOI) of 1 was comparable to the endogenous REIC/Dkk-3 level of OUMS-24 (Fig. 2A). Within a few days after infection, most of the prostate cancer cells, but not normal cells, were detached from the bottom of culture vessels. The infection efficiency of an adenovirus vector (~12% at the MOI of 0.1 and ~95% at the MOI of 1.0; Fig. 2B) and the expression level of REIC/Dkk-3 after infection with Ad-REIC (Fig. 2C) were similar between OUMS-24 and PC3 cells. To explore the cause of the detachment, we stained the cells by the TUNEL method 36 hours after the infection. As shown in Fig. 2D, many TUNEL-positive cells were observed in prostate cancer cell lines (PC3, DU145, and LNCaP) but not among normal cells (OUMS-24, PrEC, and PrSC). The incidence of TUNEL-positive cells was 49%, 24%, and 41% in PC3, DU145 and LNCaP, respectively, whereas ~1% of cells were TUNEL-positive among normal cells (Fig. 2E). A clear DNA ladder was observed in the samples prepared from PC3 cells 36 hours after the infection with Ad-REIC at MOIs >1 (Fig. 2F). These results indicate that overexpression of REIC/Dkk-3 selectively induces apoptotic cell death in prostate cancer cell lines that, for the most part, lack endogenous REIC/Dkk-3 expression.

**Mechanistic insights into the cancer cell–specific induction of apoptosis by REIC/Dkk-3.** We screened the expression levels of various apoptosis/cell cycle regulation–related proteins in PC3, the most sensitive cell line among those examined, and compared them with those in OUMS-24. Infection of PC3 cells with Ad-REIC reduced the level of antiapoptotic Bcl-2 and Bcl-xL proteins (Supplemental information; Fig. 3F). No significant change in the levels of Bax, Bad, Apaf-1, p53, p21 (CIP1/WAF1), or p16 (INK4a) was observed in either cell type. Although a caspase-8 inhibitor did not block Ad-REIC-induced apoptosis in PC3 cells (data not shown), a membrane-permeable Bax inhibitor V5 completely abrogated the apoptosis (Fig. 3A and B). Translocation of Bax protein from the cytoplasm to the mitochondria is a hallmark of triggering a Bax-mediated apoptotic pathway. Figure 3C shows that Bax protein was translocated to the mitochondria by Ad-REIC and the translocation was suppressed by V5. V5, in itself, showed no effect on the protein levels of Bcl-2 and Bax (data not shown).

One of the upstream activators of Bax is a JNK (12, 13), which promotes translocation of Bax to mitochondria (13). When we applied a JNK-specific inhibitor, SP600125, to PC3 cells, the Ad-REIC-induced apoptosis of PC3 cells was remarkably abrogated in a dose-dependent manner (Fig. 3D and E). Activation of JNK in PC3 cells infected with Ad-REIC was confirmed using a phosphorylated JNK-specific antibody and by detecting the phosphorylation of c-Jun (Fig. 3F). SP600125 is known to inhibit the kinase activity of JNK but not the phosphorylation of JNK itself. Bax protein was detected in the cytoplasm of growing PC3 cells but translocated to mitochondria in the cells infected with Ad-REIC (Fig. 3G). The mitochondrial translocation of Bax protein was associated with the release of cytochrome c into the cytoplasm and was suppressed by SP600125. JNK was partly translocated to mitochondria by Ad-REIC. These results indicate that overexpression of REIC activates JNK, reduces the Bcl-2 protein level, induces mitochondrial translocation of Bax protein, releases cytochrome c into the cytoplasm, and finally leads to apoptotic cell death. Recently, Hsieh et al. (14) reported that overexpression of REIC/Dkk-3 in human prostate cancer cell lines induces apoptosis, which is associated with the activation of the JNK and caspase-8 pathways. In our study, we observed similar results, suggesting that overexpression of REIC/Dkk-3 in prostate cancer cells could be a potential therapeutic target for cancer treatment.

**Figure 2.** Specific induction of apoptosis in human prostate cancer cell lines by overexpression of REIC/Dkk-3. **A,** expression of REIC/Dkk-3 protein in a human prostate cancer cell line PC3 36 hours after infection with an adenovirus vector carrying full-length REIC/Dkk-3 cDNA (Ad-REIC) at different MOIs. Uninfected OUMS-24 was used as a positive control. Ad-LacZ, an adenovirus vector carrying LacZ. **B,** infection efficiency of Ad-LacZ to OUMS-24 and PC3 cells as visualized by staining with X-gal. **C,** expression of REIC/Dkk-3 in OUMS-24 and PC3 cells after infection with Ad-REIC at 10 MOI. D, TUNEL staining (green) of normal human cells (OUMS-24, PrEC, and ProSc) and prostate cancer cell lines (PC3, DU145, and LNCaP) fixed 36 hours after the infection at 10 MOI. Insets, cells stained with 4’,6-diamidino-2-phenylindole (blue). E, percentage of TUNEL-positive cells counted under the same conditions as those in (D). F, characteristic fragmentation of DNA was observed in PC3 cells infected with Ad-REIC at MOIs >1. One day after plating of 5 × 10⁵ PC3 cells, the cells were infected with the indicated virus vectors at various MOIs and harvested 36 hours later.
of REIC/Dkk-3 induced apoptotic cell death in several types of human cancer cell through activation of caspase-3, which is known to be a major apoptosis executor in the down-streaming of cytochrome c.

At present, it is not clear whether JNK activated by Ad-REIC acts on Bcl-2 and Bax directly or indirectly, although Bcl-2 (15), Bcl-xL (16), and Bim (17) have been identified as targets of both JNK and c-Jun. We confirmed that the REIC/Dkk-3 protein was glycosylated and secreted into the culture medium when overexpressed in PC3 cells. The amount and intracellular localization of ß-catenin were not affected by Ad-REIC (Fig. 3F; data not shown), excluding the possibility of involvement of the canonical pathway of Wnt signaling. JNK may be activated through the noncanonical pathway of Wnt signaling by secreted REIC/Dkk-3; more likely, it is activated intracellularly through a stress-sensing system for the overexpressed protein or through yet unknown intracellular target proteins of REIC/Dkk-3. In this context, it should be noted that md-7/IL-24, which also selectively induces apoptosis in many different types of human cancer cells, efficiently induced apoptosis in human prostate cancer cell lines even when the secretion was blocked by truncating the signal peptide (17). Although Bax is activated by p53 under certain conditions (18), induction of apoptosis by Ad-REIC is apparently independent of p53 function because PC3 is null in p53 (Supplemental information; ref. 19). This is true also of md-7/IL-24, which exerts its effect through p53-independent activation of Bax (20). Recently, Hoang et al. (5) reported that overexpression of REIC/Dkk-3 did not induce apoptosis in a human osteosarcoma cell line, Saos-2, but inhibited invasion and motility of the cells in vitro.4 REIC/Dkk-3 may exert its anticancer activity at different action points.

**REIC/Dkk-3-targeted gene therapy for prostate cancer in a xenotransplantation model.** Because selective growth inhibition and apoptosis by overexpression of REIC/Dkk-3 were indicated by in vitro studies, we investigated the effect of Ad-REIC on the growth of PC3 cells in vivo. PC3 cells of 2.5 × 10^6 in Matrigel were transplanted s.c. into nude mice. One week after the transplantation, when the tumor volume reached 30 to 100 mm^3, 2.5 × 10^7 plaque-forming units of Ad-REIC or Ad-LacZ in 100 µL were injected intratumorally. The tumors progressively grew in the buffer-injected control and Ad-LacZ-injected groups during

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4 In accord with our unpublished data which was obtained under similar conditions.
Figure 4. Effect of Ad-REIC on the growth of PC3 in nude mice. A, appearance of tumors at the end of the observation period. *, mean volume of tumors estimated from the diameters in five nude mice in each group. PBS; ■, Ad-LacZ; ●, Ad-REIC; vertical bars, SD. Insets, volume of each tumor in the nude mice injected with Ad-REIC. Four of the five mice were completely tumor-free when autopsied 30 days after the virus injection. C, TUNEL staining of tumor tissues obtained on autopsy 30 days after the virus injection. PI, stained with propidium iodide to visualize nuclei.

the observation period of 1 month (Fig. 4A and B). In contrast, the tumors completely disappeared in four out of five mice in the group receiving Ad-REIC injection; even in the tumor-bearing mouse in this group, the tumor did not actively grow and remained unchanged throughout the observation period. An ~2-fold increase in tumor size was observed in two of the five transplanted tumors during the first week, possibly due to the lag time until the availability of fully functional REIC/Dkk-3 protein and local effects of injection, including edema and inflammation. The tumors were resected at the end of observation and examined by TUNEL staining (Fig. 4C). No apoptotic cells were observed in tumors injected with the buffer or with Ad-LacZ, whereas many cells were positive in TUNEL staining even 1 month after the injection of Ad-REIC in the residual tumor.

The sharply selective induction of apoptosis in culture that was observed and the highly efficient inhibition of tumor growth in vivo by overexpression of REIC/Dkk-3 implies extraordinarily promising characteristics of REIC/Dkk-3 as a target gene for cancer therapy, possibly comparable to p53 and mda-7/IL-24 (17). Prostate cancer is the most commonly diagnosed malignancy in many Western countries. Various therapeutic measures including anti-androgen therapy have been applied to prostate cancer with considerable success. However, once prostate cancers acquire androgen-independent growth capabilities at later stages, as did PC3, they are hardly controlled by the conventional therapies and often exhibit lethality. It is hoped that our present results lead to the identification of a new molecular target for counteracting this notoriously vicious disease.

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References

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