Acta Medica Okayama

Mechanistic Analysis of Resistance to REIC/Dkk-3-induced Apoptosis in Human Bladder Cancer Cells

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Abstract

We have recently shown that a new therapeutic modality using the REIC/Dkk-3 gene (Ad-REIC) is effective against various human cancers, including those of prostate, testis and breast origins. The aim of the present study was to examine the sensitivity of bladder cancers to Ad-REIC and to clarify the molecular mechanisms that determine sensitivity/resistance. We found that 2 human bladder cancer cell lines, T24 and J82, are resistant to Ad-REIC. In T24 and J82 cells, the ER stress response and activation of JNK were observed in a manner similar to that in the sensitive PC3 cells. Translocation of Bax to mitochondria occurred in PC3 cells but not in T24 and J82 cells. Bcl-2 was remarkably overexpressed in T24 and J82 compared with the expression levels in sensitive cell lines. Treatment of T24 and J82 cells with a Bcl-2 inhibitor sensitized the cells to Ad-REIC-induced apoptosis. The results indicate that some human bladder cancers are resistant to apoptosis induced by overexpression of REIC/Dkk-3, which is at least in part due to up-regulation of Bcl-2. These results provide a basis for possible use of Bcl-2 as a marker of sensitive cancers and to try to sensitize resistant cancers to Ad-REIC by down-regulation of Bcl-2.

KEYWORDS: REIC/Dkk-3, bladder cancer, apoptosis, Bcl-2

Original Article

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We have recently shown that a new therapeutic modality using the REIC/Dkk-3 gene (Ad-REIC) is effective against various human cancers, including those of prostate, testis and breast origins. The aim of the present study was to examine the sensitivity of bladder cancers to Ad-REIC and to clarify the molecular mechanisms that determine sensitivity/resistance. We found that 2 human bladder cancer cell lines, T24 and J82, are resistant to Ad-REIC. In T24 and J82 cells, the ER stress response and activation of JNK were observed in a manner similar to that in the sensitive PC3 cells. Translocation of Bax to mitochondria occurred in PC3 cells but not in T24 and J82 cells. Bcl-2 was remarkably overexpressed in T24 and J82 compared with the expression levels in sensitive cell lines. Treatment of T24 and J82 cells with a Bcl-2 inhibitor sensitized the cells to Ad-REIC-induced apoptosis. The results indicate that some human bladder cancers are resistant to apoptosis induced by overexpression of REIC/Dkk-3, which is at least in part due to up-regulation of Bcl-2. These results provide a basis for possible use of Bcl-2 as a marker of sensitive cancers and to try to sensitize resistant cancers to Ad-REIC by down-regulation of Bcl-2.

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I nvasive bladder cancer is a potentially fatal disease. Approximately 30% of bladder cancer cases are invasive at initial diagnosis, and 10–15% of superficial bladder cancer cases progress to invasive cancer during the disease course [1]. When the cancer is confined to the bladder, radical cystectomy combined with pelvic lymphadenectomy results in a 5-year survival rate of approximately 75% with no appreciable tumor progression. It is difficult, however, to detect lymphatic micrometastasis in remote tissues prior to surgical resection, and lymphatic micrometastasis leads to poor prognosis with a progression-free 5-year survival rate of only 25–30% due to recurrence of cancer [2]. Postoperative recurrence of bladder cancer is often found in remote lymph nodes and/or organs rather than in neighboring tissues of the bladder, indicating that remote micrometastasis is critical for eventual recurrence. Neoadjuvant chemotherapy using cisplatin, methotrexate, vinblastine and doxorubicine has been conducted, but the difference in 5-year survival after surgical resection alone and that after surgical resection combined with neoadjuvant chemotherapy remains statistically elusive ($p = 0.06$) [3]. In addition, the side effects of systemic

application of anti-cancer drugs remain a major challenge despite the development of new drugs and improvements in regimens. Patients with invasive bladder cancer often suffer from irreversible renal insufficiency due to urinary retention or obstruction of the ureter. Renal insufficiency is another complicating factor preventing well-controlled application of chemotherapeutic agents.

Gene therapy is a promising new approach that may circumvent the problems described above. A number of different genes, including p53, HSV-derived thymidine kinase and TRAIL, have been intensively exploited as potential therapeutic modalities [4–6]. We have recently reported that REIC/Dkk-3, a newly identified tumor suppressor gene [7, 8], might be useful for gene therapy. Expression of REIC/Dkk-3 was found to be reduced in many different types of human cancers [8–13], mainly due to hypermethylation of the promoter region [14]. When overexpressed using an adenovirus vector (Ad-REIC), REIC/Dkk-3 induced apoptosis in prostate cancer cells but not in normal prostate epithelial cells, despite similar expression levels in both cells [9]. A single injection of Ad-REIC had a dramatic anti-tumor effect on a xenotransplanted human prostate cancer. In addition, Ad-REIC was shown not only to induce apoptosis but also to inhibit local invasion and metastasis in an orthotopic prostate cancer model in mice [10]. Ad-REIC is also effective for human testicular cancer [11].

The original idea of using a tumor suppressor gene as a tool for gene therapy is to correct the deteriorated function of the corresponding tumor suppressor genes in cancer cells. Although REIC/Dkk-3 is a secretory protein and is assumed to exert its physiological function from the cell surface, the induction of apoptosis in cancer cells by Ad-REIC has been shown to be primarily due to ER stress loaded by overproduction of REIC/Dkk-3 in the cells [9, 11]. Activation of c-Jun N-terminal kinase (JNK) triggered by ER stress is a critical event for apoptosis induced by Ad-REIC.

Although Ad-REIC has been shown to be effective to many different types of human cancers, we have also noted that not all human cancers are sensitive to Ad-REIC. It is important, therefore, to determine factors that are responsible for the sensitivity/resistance of cancer cells to Ad-REIC in order to select

cases in which Ad-REIC can be used and to develop strategies for overcoming resistance. In the present study, we identified 2 human bladder cancer cell lines that are resistant to Ad-REIC and we analyzed the intracellular signal transduction pathway triggered by Ad-REIC in the resistant cell lines compared to that in sensitive control cell lines. We found that overexpression of Bcl-2 is a factor conferring resistance of cells to Ad-REIC-induced apoptosis.

Materials and Methods

Reagents, cells, and culture.   Human transitional cell carcinoma (TCC) cell lines derived from the bladder, including T24 and J82, the prostate cancer cell line PC3, the osteosarcoma cell line Saos2, and the germ cell tumor NCCIT were purchased from ATCC (Rockville, MD, USA). A normal human embryonic fibroblast line, OUMS-24, was established and provided by Dr. M. Namba [15]. McCoy's 5A medium, MEM, Ham's F-12 K medium, and Dulbecco's modified MEM were used for T24, J82, PC3, and OUMS-24, respectively, with a supplement of 10% FBS and antibiotics. Saos2 and NCCIT were maintained in RPMI1640 supplemented with 10% fetal bovine serum. Subfractionation of cells was performed using a Mitochondrial Fractionation kit (Active Motif, Carlsbad, CA, USA). A Bcl-2 inhibitor, YC-137, was purchased from CALBIOCHEM (San Diego, CA, USA).

RT-PCR.   A full-length cDNA of REIC/Dkk-3 was integrated into the cosmid vector pAxCAwt and transferred into an adenovirus vector (Ad-REIC) by the COS-TPC method (TAKARA BIO, Shiga, Japan). An adenovirus vector carrying the LacZ gene (Ad-LacZ) was used as a control. The virus vectors were amplified in HEK 293 cells and purified using an Adeno-X Virus Purification kit (Clontech Laboratories, Mountain View, CA, USA).

Adenovirus vector carrying REIC/Dkk-3. To determine the infection efficiency, cells were infected with Ad-LacZ. Twenty-four hours after infection, the cells were washed with PBS, fixed at 4℃ for 3 min in 0.2% glutaraldehyde and 1% formaldehyde, rinsed twice with PBS, and then stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal [BRL, Gaithersburg]) at 37℃ for 12 h.

Cytotoxicity/apoptosis assay.   Cell viability

was assessed using a LIVE/DEAD® Cell viability Fluorometric (green/red) Assay kit (Molecular Probes). Dead cells were identified as those stained red. Alternatively, cells with shrunken or fragmented nuclei, as demonstrated by staining with Hoechst 33342, were regarded as apoptotic cells.

Western blot analysis.   Forty-eight h after adenoviral infection, cells were washed in PBS, fixed at 4℃ in 10% trichloroacetic acid, harvested, and lysed in an 8M urea solution. Extracted protein samples were separated by gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membranes. After blocking in 10% skim milk, the membranes were incubated overnight with primary antibodies at 4℃. The membranes were washed with PBS, incubated with secondary antibodies at room temperature for 1h, and rinsed with PBS. Signals were visualized using ECL Plus™ (Amersham Biosciences). The antibodies used were as follows: rabbit anti-human REIC/Dkk-3 antibody raised in our laboratory; Apoptosis Sampler I kit (BD Biosciences) for Bcl-2, Bcl-xL, Nip, and XIAP; rabbit anti-human Bax antibody (Upstate Cell Signaling Solutions); mouse anti-human tubulin antibody (Sigma); mouse anti-horse cytochrome c antibody (Upstate Biotechnology, Lake Placid, NY, USA); mouse anti-mouse mitochondrial Hsp70 (Abcam, Cambridge, UK); rabbit anti-human Hsp27 antibody (Santa Cruz); rabbit anti-human Hsp40 antibody (Stressgen Bioreagents, British Columbia, Canada); rabbit anti-human c-Jun antibody, rabbit anti-human phospho-c-Jun (Ser63) antibody, rabbit anti-human JNK (c-Jun N-terminal kinase) antibody, rabbit anti-human phos-pho-JNK (Thr183/Tyr185) antibody, and rabbit anti-human Hsp70/73 antibody (Cell Signaling Technology, Beverly, MA, USA).

Electromobility shift assay of a target element of ATF-6.   For an electromobility shift assay, a cis-acting endoplasmic reticulum (ER) stress response element (ERSE: 5′–CCAATCAGAAAGT GGCACG–3′) in the human CHOP promoter was used as a DNA probe [16]. The double-stranded probe (0.5 μg) was labeled with 32P in the presence of 10μ Ci of [γ-32P]-ATP (Institute of Isotopes of Hungarian Academy of Sciences: HAS, Budapest, Hungary) and T4 polynucleotide kinase (New England Biolabs: NEB, Tozer Road Beverly, MA, USA). The electromobility shift assay was carried out by
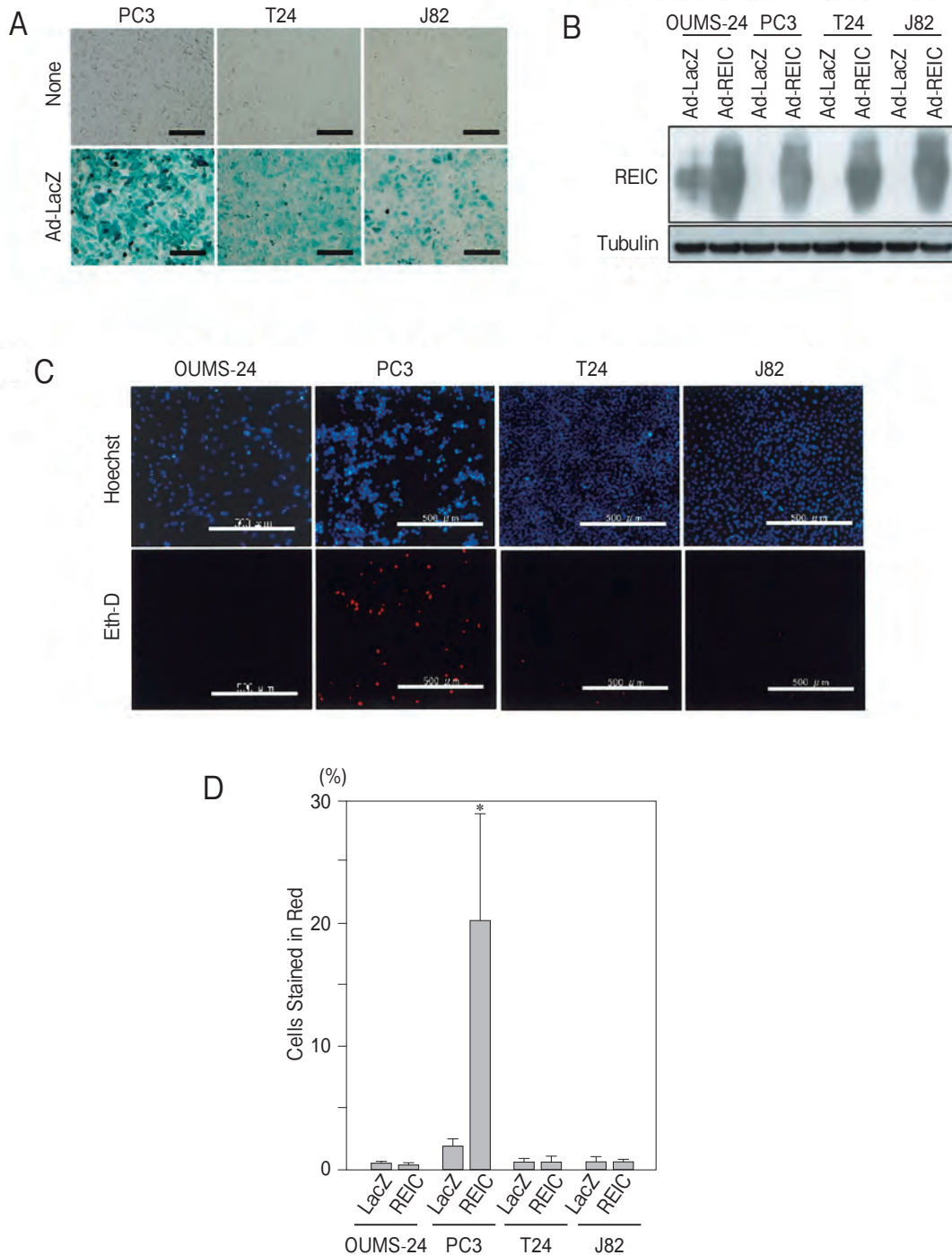
adding 2μg of crude nuclear extracts of cells to each reaction mixture (20μl) containing 2.5ng of the labeled probe, 60mM KCl, 5mM MgCl2, 0.1mM EDTA, 1μg poly (dI-dC), 1mM dithiothreitol (DTT), 5% glycerol, and 20mM Hepes/pH 7.9. For a super shift experiment, 2μg of a rabbit anti-human ATF6 antibody (Abcam, Cambridge, UK) or of a control rabbit IgG (Vector Laboratories, Burlingame, CA, USA) was added to the reaction mixture. DNA/protein complexes were then separated by electrophoresis in a 5% polyacrilamide gel under non-denaturing conditions.

Results

Resistance of human bladder cancer cell lines to Ad-REIC-induced apoptosis.   We first examined the endogenous protein levels of REIC/Dkk-3 in human TCC cell lines. All of the six TCC cell lines examined showed remarkably low or undetectable levels of REIC/Dkk-3, as demonstrated by RT-PCR and Western blot analysis (data not shown). Among the cell lines, we chose T24 and J82 for further analyses since these cell lines were shown to be relatively remote in their gene expression profile [17].

The infection efficiency of Ad-REIC to T24 and J82 cells was similar to that to PC3 as assessed using Ad-LacZ (Fig. 1A). Furthermore, the expression levels of REIC/Dkk-3 protein in T24 or J82 cells infected with Ad-REIC were similar to those in OUMS-24 and PC3 cells (Fig. 1B). Apoptotic cell death, however, was induced by Ad-REIC in PC3 cells but not in either T24 cells or J82 cells (Figs. 1C and 1D). This result was unexpected since cancer cells lacking endogenous expression of REIC/Dkk-3 were mostly sensitive to Ad-REIC. We therefore analyzed the intracellular signal transduction pathway triggered by Ad-REIC.

Signal transduction triggered by Ad-REIC in human bladder cancer cell lines.   In our previous studies [9, 11, 18], the triggering event leading to apoptosis by Ad-REIC was shown to be ER stress. Activation of JNK was shown to be the essential downstream step, followed by translocation of Bax protein from the cytoplasm to mitochondria and eventual release of cytochrome C into the cytoplasm. ER stress by overexpression of REIC/Dkk-3 was more directly confirmed by an electromobility shift assay,

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Fig. 1   Transitional cell carcinoma cell lines were resistant to apoptosis induced by Ad-REIC. **A**, Infection efficiency of Ad-LacZ (20 MOI) in human transitional carcinoma cell lines, T24 and J82. PC3, a human prostate cancer cell line, was used as a positive control. Successfully infected cells were identified by intracellular β-galactosidase activity (in blue) 24h after infection. Scale bars, 50μm; **B**, Overexpression of REIC/Dkk-3 using an adenovirus vector (Ad-REIC). Ad-LacZ was used as a control. OUMS-24 cells, normal human fibroblasts, were used as a control; **C**, the viability of cells examined 48h after infection with Ad-REIC at 20 MOI. Cell nuclei were stained blue by Hoechst 33342. Dead cells were stained red by an ethidium bromide dimer (Eth-D). OUMS-24 and PC3 cells were used as negative and positive controls, respectively. Scale bars, 500μm; **D**, Quantitation of apoptotic cells shown in **A**. Vertical bars indicate the standard deviation. $^{*}p < 0.05$.

which showed that ATF6, a well-known sensor molecule for ER stress [19], was activated to bind to an ER stress responsive element in the CHOP promoter (Fig. 2A). Expression of CHOP was induced in PC3, T24 and J82 cells but not in OUMS-24 cells (Fig. 2B). Activation of JNK demonstrated by phosphorylation of JNK itself and of the substrate c-Jun was also observed in the 3 cancer cell lines but not in OUMS-24 cells. Translocation of Bax to and release of cytochrome C from mitochondria were observed in the sensitive PC3 cells but not in the T24 and J82 cells that were resistant to Ad-REIC-induced apoptosis (Fig. 2C). These results indicate that there was interference with a signal transduction step between activation of JNK and translocation of Bax in the resistant T24 and J82 cells.

Overexpression of Bcl-2 as a cause of resistance to Ad-REIC-induced apoptosis. We next screened expression of apoptosis-related proteins and heat shock proteins (Fig. 3). NCCIT and Saos2 were included for the analysis as cell lines sensitive to and resistant to Ad-REIC-induced apoptosis. Except for Hsp 40 and Nip, the expression levels of the proteins varied among cell lines. The expression levels of Bcl-2 were most notable, since the protein was overexpressed in the 3 resistant cancer cell lines in common compared with the expression levels of Bcl-2 in the 2 sensitive cancer cell lines. Overexpression of anti-apoptotic Bcl-xL and down-regulation of proapoptotic XIAP in T24 and J82 cells were also noted.

If overexpression of Bcl-2 is responsible for the resistance of cells to Ad-REIC-induced apoptosis, inhibition of Bcl-2 should restore the sensitivity. As shown in Fig. 4, apoptosis was induced in T24 and J82 cells by infection with Ad-REIC when the cells were treated in advance with the Bcl-2 inhibitor YC137.

Discussion

REIC/Dkk-3 was first identified as a gene whose expression is reduced in immortalized cell lines compared to that in their normal counterparts and was subsequently shown to be a candidate tumor suppressor gene [8]. Expression of the REIC/Dkk-3 gene was later shown to be reduced in many human cancer cells and tissues, including prostate cancer, renal clear cell carcinoma, non-small cell lung caner, and

testicular germ cell tumor [9, 11–13]. Forced expression of REIC/Dkk-3 using an adenovirus vector was found to induce apoptotic cell death in REIC/Dkk-3-deficient cancer cells but not in REIC/Dkk-3-proficient normal cells [9, 11, 18]. This result was confirmed in transplanted cancer cells *in vivo*.

Our previous studies have indicated that Ad-REIC induces apoptotic cell death through ER stress and not by physiological functions as a secreted protein. Mda-7/IL-24 is one of the most potent and promising gene therapeutic agents against various human cancers [20]. Recently, mda-7 was shown to exert its tumor-specific cell-killing effect at least partly through ER stress [21]. Application of purified REIC/Dkk-3 protein from the culture medium did not affect the survival of any of the cancer cell lines examined (our unpublished results). Overexpression of non-functional partial peptides of REIC/Dkk-3 also induced apoptosis in PC3 cells (manuscript submitted). ATF6, a sensor protein of ER stress [19], was activated and CHOP, an indicator gene of ER stress [22], was induced in the cancer cell lines examined (Figs. 2A and 2B). CHOP, one of the target genes of activated ATF6 [16], was not induced to any appreciable levels in OUMS-24 cells, although activation of ATF was observed in an electromobility shift assay. This result may have been due to the presence of a factor(s) suppressing the expression of CHOP in OUMS-24 cells or due to the involvement of some other pathways in the induction of CHOP [23].

Activation of JNK has been shown to be essential for Ad-REIC-induced apoptosis [9]. Treatment with an inhibitor of JNK and with an inhibitor of the downstream effector Bax abrogated the apoptotic cell death in PC3 cells. In T24 and J82 cells, activation of JNK but not translocation of Bax from the cytoplasm to mitochondria was observed upon infection with Ad-REIC (Figs. 2B and 2C), indicating that a step from JNK to Bax is a branching point of cell survival or cell death. Bcl-2 family proteins are characterized by containing at least one of the four Bcl-2 homology domains (BH1-BH4). Some of the member proteins, including Bcl-2 and Bcl-xL, function as typical anti-apoptotic proteins, while other members such as Bax and Bak promote apoptosis. It is thought that Bcl-2 dimerizes with Bax through its BH1 and BH2 domains and thereby inhibits the function of Bax to destabilize the outer mitochondrial membrane [24]. The ratio of
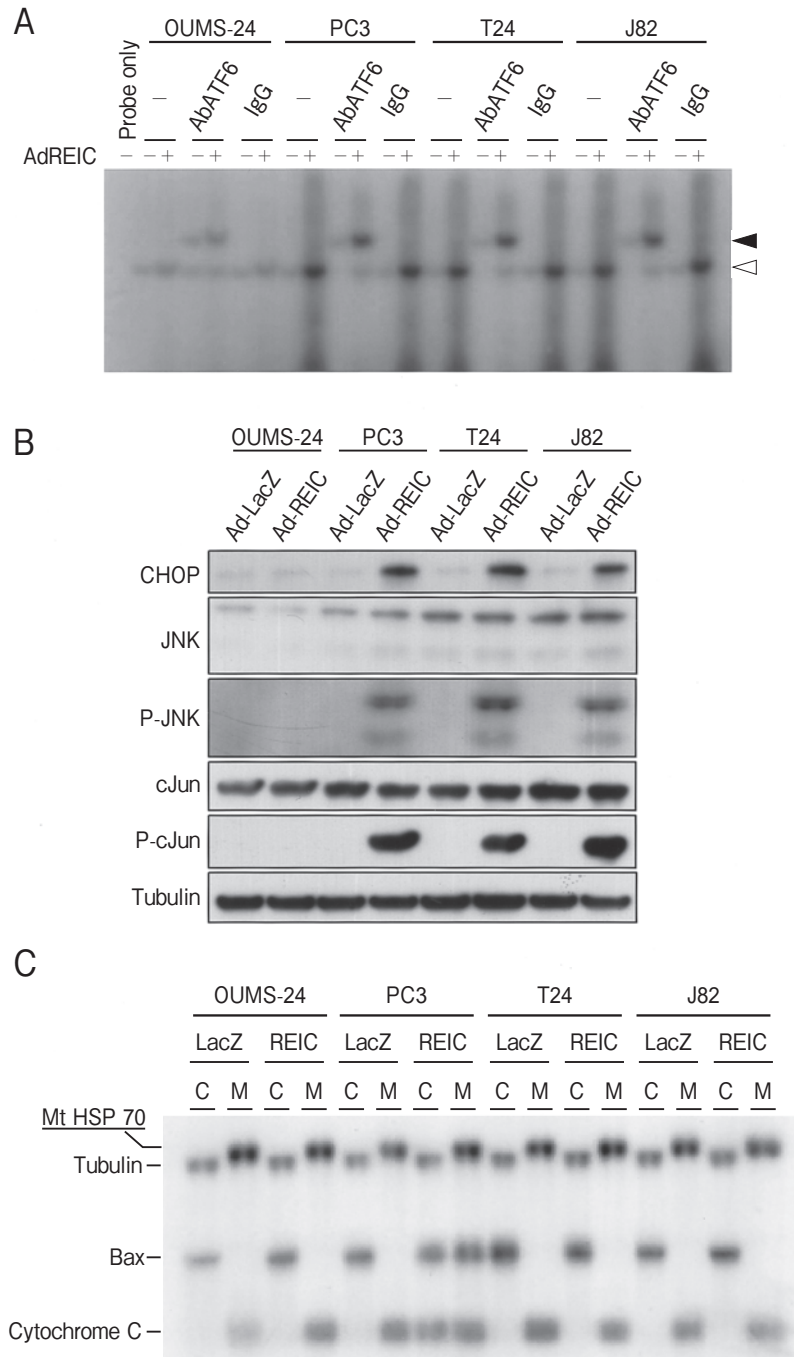
Fig. 2   Signal transduction pathway triggered by Ad-REIC and leading to apoptosis. **A**, Electromobility shift assay of an ER stress response element (ERSE: 5'‒CCAATCAGAAAGTGGCACG‒3') in the human CHOP promoter, a target of ATF6 activated by ER stress. Nuclear extracts were prepared from cells infected with Ad-REIC at 20 MOI at 42 h. For a super shift experiment, a rabbit anti-human ATF6 antibody was added to the reaction mixtures. Open arrowhead, shifted probes; black arrowhead, super-shifted probes; **B**, Western blot analysis for proteins involved in the signal transduction. Cells were treated under the same conditions as those described in A. P-JNK and P-cJun are phosphorylated JNK and cJun. Tubulin was used as a control for loaded amounts of protein. LacZ and REIC indicate infection with Ad-LacZ and with Ad-REIC, respectively; **C**, Western blot analysis for proteins involved in the signal transduction performed using a mixture of designated antibodies. Mt (mitochondrial) HSP70 and tubulin were used as markers for mitochondrial and cytoplasmic fractions, respectively.
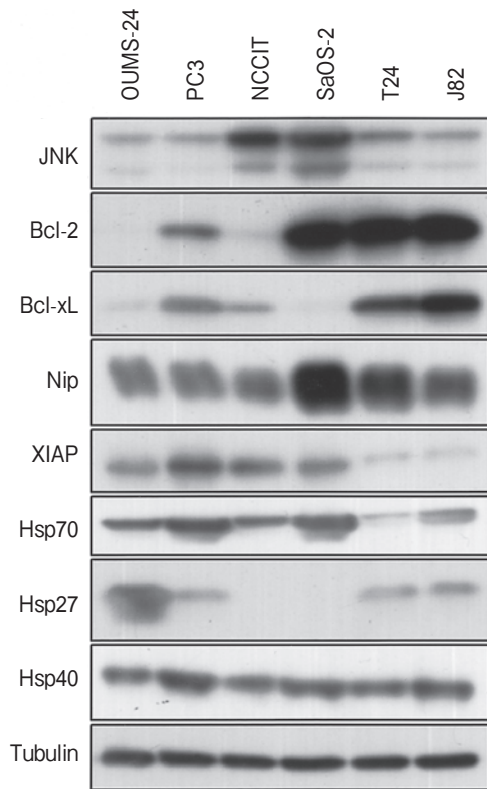
Fig. 3   Western blot analysis for apoptosis-related proteins and heat shock proteins in various cell lines. Tubulin was used as a control for the loaded amounts of proteins.

Bcl-2 to Bax dictates susceptibility to an apoptotic stimulus [24]. It is conceivable, therefore, that overexpression of Bcl-2 consistently observed in the cancer cell lines inert to Ad-REIC-induced apoptosis is the mechanistic cause for the resistance. YC137 interferes with Bcl-2 function by blocking protein-protein interaction via the SH3 domain. Treatment of T24 and J82 cells with YC137 restored the sensitivity of those cells to Ad-REIC (Fig. 4).

The functional significance of Bcl-2 has been extensively investigated both in basic and clinical studies on urothelial carcinomas, and clinical oncologists have shown interest in Bcl-2 not only as a biomarker but also as a target of molecular therapy. Overexpression of Bcl-2 has been shown to correlate to varying degrees with poor prognosis and early recurrence of bladder cancer [25–27] and with resistance to chemotherapy and gene therapy [28, 29]. Down-regulation of Bcl-2 using antisense oligonucleotides at least partially sensitizes bladder cancers to cisplatin and radiotherapy [30, 31]. The results of the present study indicate that overexpression of Bcl-2 confers upon some bladder cancer cell lines at least partial resistance to gene therapy using Ad-REIC, which has been shown to be a very potent tumor-specific modality
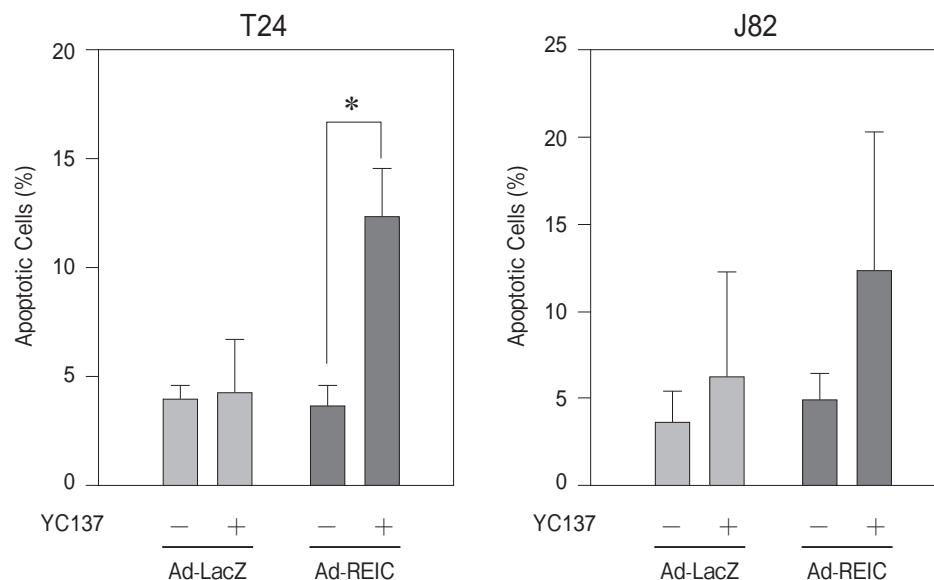
Fig. 4   Sensitization of T24 and J82 cells to Ad-REIC-induced apoptotic cell death by a Bcl-2 inhibitor. The cells were treated under conditions similar to those described in the legend to Fig. 1. YC137 (0.5 mM) was added 36 h after infection. At 42 h and 54 h after infection, apoptotic cells with shrunken or fragmented nuclei were determined after staining with Hoechst33342. Vertical bars indicate the standard deviation. $^*p < 0.05$

against many other cancers. It has been reported that not all human bladder cancer cells overexpress Bcl-2 [25–27]. We therefore should examine the possibility of diagnosing sensitive bladder cancer cases using expression of Bcl-2 as a marker and try to sensitize bladder cancer cells to Ad-REIC by down-regulation of Bcl-2 using siRNA, miRNA and low-molecular-weight substances as well as oligonucleotides. The results of the present study provide a basis for such lines of research, which will eventually lead to the development of more potent and patient-friendly therapeutic measures.

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