Small-interfering RNA–induced androgen receptor silencing leads to apoptotic cell death in prostate cancer

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Abstract
Prostate cancer is the second leading cause of cancer death in the United States and, thus far, there has been no effective therapy for the treatment of hormone-refractory disease. Recently, the androgen receptor (AR) has been shown to play a critical role in the development and progression of the disease. In this report, we showed that knocking down the AR protein level by a small interfering RNA (siRNA) approach resulted in a significant apoptotic cell death as evidenced by an increased annexin V binding, reduced mitochondrial potential, caspase-3/6 activation, and DFF45 and poly(ADP-ribose) polymerase cleavage. The apoptotic response was specifically observed in those siRNA-transfected cells that harbor a native AR gene. No cell death was found in the AR-null prostate cancer cell PC-3 or its subline that has been reconstituted with an exogenous AR gene, as well as two breast cancer cell lines that are AR positive. Moreover, in parallel with the siRNA-induced AR silencing, the antiapoptotic protein Bcl-xL was significantly reduced, which might account for the apoptotic cell death because ectopic enforced expression of Bcl-xL partially inhibited apoptosis after AR silencing. Taken together, our data showed that knocking down the AR protein level in prostate cancer cells leads to apoptosis by disrupting the Bcl-xL–mediated survival signal downstream of AR-dependent survival pathway. [Mol Cancer Ther 2005; 4(4):505–15]

Introduction
Prostate cancer is a significant risk for men in the United States (1). Sixty years ago, it was found that androgens were required for prostate epithelial cells to proliferate, differentiate, and survive; apoptotic cell death has been found in the prostate after androgen withdrawal (2, 3). Because of this insight, androgen ablation has been widely accepted as a major medical treatment for metastatic prostate cancer. However, most patients treated by androgen ablation ultimately relapse to more aggressive incurable hormone refractory prostate cancer (4). Moreover, antiandrogen withdrawal syndrome is another concern for androgen antagonist therapy (5). The etiology of hormone-refractory relapse may have various molecular causes, but in each scenario the androgen receptor (AR) is expressed and its function is maintained (6–11), suggesting that androgen-independent AR signaling is involved. In a transgenic mouse model, AR overexpression in prostate epithelium resulted in marked increases in epithelial proliferation and focal areas of intraepithelial neoplasia in the ventral prostate and dorsolateral prostate (12). Recently, the critical role of the AR for cellular proliferation in vitro or tumor growth in vivo of prostate cancer has been shown by different approaches, including disruption of AR function by anti-AR antibody, inhibition of AR expression by AR-specific ribozyme or antisense oligonucleotides, as well as knocking down AR expression by the RNA interference (RNAi) approach (8, 13–15). However, the mechanisms of AR-dependent cellular survival remain unclear in prostate cancer progression although some survival mechanisms involved in hormone-resistant progression of prostate cancer have been proposed (16–20).

Apoptosis, or programmed cell death, is a well-conserved process whose basic tenets remain common to all metazoans (21, 22). Intracellular organelles, like mitochondria, are key participants in apoptosis. The main aspects of mitochondrial involvement in apoptotic process include two critical events, the onset of multiple parameters of mitochondrial dysfunction, such as loss of membrane potential and the release of mitochondrial proteins including cytochrome c. The Bcl-2 family proteins are critical regulators that directly control the mitochondrial function and consist of both proapoptotic and antiapoptotic members (23). Bax, Bak, and Bok are proapoptotic members, as are the BH3 domain only members, such as Bad, Bik, and Bid. Antiapoptotic members include Bcl-2 and Bcl-xL, Bcl-w, Mcl-1, etc. It is believed that the relative levels of proapoptotic and antiapoptotic members are the key determinants in the regulation of cell death and survival.
The bcl-x gene encodes multiple spliced mRNAs, of which Bcl-xL is the major transcript (23, 24). Like Bcl-2, Bcl-xL protects cells from apoptosis by regulating mitochondrial membrane potential and volume, and subsequently prevents the release of cytochrome c and other mitochondrial factors from the intermembrane space into cytoplasm. In addition, Bcl-xL may prevent apoptosis via a cytochrome c-independent pathway (25). Although Bcl-xL protein can be regulated posttranslationally, it is mainly controlled at the gene expression level (26, 27). Bcl-xL protein is detected in the epithelial cells of normal prostate gland and prostate cancers in an earlier report (28). The expression level of Bcl-xL protein correlated with higher grade and stage of the disease, indicating an important role of Bcl-xL in prostate cancer progression.

RNAi is a recently discovered mechanism of posttranscriptional gene silencing in which double-stranded RNA corresponding to a gene (or coding region) of interest is introduced into an organism, resulting in degradation of the corresponding mRNA (29, 30). Unlike antisense technology, the RNAi phenomenon persists for multiple cell divisions before gene expression is regained, and is more efficient than antisense oligonucleotides. RNAi is, therefore, an extremely powerful, simple method for assaying gene function (31).

In an effort to dissect the mechanism of AR-dependent survival and to develop novel therapeutic strategies for prostate cancer, we knocked down the AR protein expression in prostate cancer cells that harbor the AR gene using the RNAi technique. Surprisingly, in addition to cell arrest, we found a significant apoptotic cell death when AR expression was knocked down by a small interfering RNA (siRNA) duplex. Most interestingly, the antiapoptotic protein Bcl-xL was also decreased in parallel with AR silencing, and overexpression of exogenous Bcl-xL controlled by a cytomegalovirus promoter partially rescued the cells from AR siRNA-induced apoptosis.

Materials and Methods

**Cell Lines and Reagents**

The human prostate cancer LNCaP, LAPC-4, PC-3, C4-2 and CWR22Rv1 cells, and HEK293 cells were described previously (32–34). The cell line information is briefly summarized in Table 1. Prostate epithelial cell RWPE-1 and breast cancer cell lines MCF-7 and T47D were obtained from American Type Culture Collection (Manassas, VA). The hormone-refractory prostate cancer LNCaP-Rf was a kind gift provided by Dr. Donald Tindall (Department of Biochemistry, Mayo Clinic, Rochester, MN; ref. 13). PC-3/AR subline was established by stably transfecting the AR-null PC-3 cells with a vector bearing the human AR gene obtained from Dr. Fahri Saatcioglu (Department of Biology, University of Oslo, Oslo, Norway). PC-3/Neo subline was established when an empty vector was used. The stable clones were selected in a puromycin-containing culture medium. Antibodies against human AR, actin, and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against caspases, cytochrome c, Bcl-2 family members, XIAP, DFF45, and poly(ADP-ribose) polymerase were obtained from Cell Signaling (Beverly, MA). JC-1 fluorescent dye was obtained from Molecular Probes (Eugene, OR). Charcoal-stripped FBS was obtained from Atlanta Biologicals (Norcross, GA). Other reagents were supplied by Sigma (St. Louis, MO).

**siRNA Synthesis, Labeling, and Transfection**

Sequence information regarding the human AR gene (Genbank accession no. NM_000044) was extracted from the National Center for Biotechnology Information Entrez nucleotide database. Up to 34 mRNA segments were identified using the OligoEngine software (OligoEngine, Inc., Seattle, WA), which fulfill the requirements for potentially triggering RNAi according to the literature (31). The AR gene specificity was confirmed by searching the National Center for Biotechnology Information BlastN database. The siRNAs were prepared by a transcription-based method using the Silencer siRNA construction kit (Ambion, Austin, TX) according to the manufacturer’s instructions. The 29-mer sense and antisense DNA oligonucleotide templates (21 nucleotides specific to the targets and 8 nucleotides specific to T7 promoter primer sequence 5′- CCTGTCTC-3′) were synthesized by IDT (Coralville, IA). The quality of the synthesized siRNA was estimated by agarose gel analysis and found to be very clean. RNAs were quantified by using Ribogreen fluorescence (Molecular Probes). A Silencer siRNA labeling kit using a fluorescent Cy3 dye (Ambion) was used for labeling the siRNA duplexes according to the manufacturer’s instructions. The purified siRNA duplexes were transfected into cells with the Oligofectamine reagent (Invitrogen, Co., Carlsbad, CA) in a medium supplied with 2% charcoal-stripped FBS. The media were changed every 3 days. A scrambled negative siRNA duplex (Ambion) was used as control. A pooled chemically synthesized AR siRNA mixture was purchased from Upstate (Charlottesville, VA).

**Western Blotting and Immunofluorescence Staining**

For Western blot, cells were washed in PBS and lysed in a radioimmunoprecipitation assay buffer supplied with protease inhibitors (CytoSignal, Irvine, CA). Western blot analysis was done as described previously (32–35) to assess the protein expression level of target molecules. Blots were developed with a SuperSignal West Dura substrate kit (Pierce Biotech, Rockford, IL). Immunofluorescent staining was done as previously described (34, 35). The picture was taken under a fluorescence microscope (Nikon Inc., Melville, NY) set at ×200 magnification.
Table 1. Summary of cell lines used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin and modification</th>
<th>AR status</th>
<th>Hormone response</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>Human prostate cancer</td>
<td>Mutant</td>
<td>Yes</td>
<td>55</td>
</tr>
<tr>
<td>LAPC-4</td>
<td>Human prostate cancer</td>
<td>Wild type</td>
<td>Yes</td>
<td>56</td>
</tr>
<tr>
<td>PC-3</td>
<td>Human prostate cancer</td>
<td>Null</td>
<td>No</td>
<td>57</td>
</tr>
<tr>
<td>C4-2</td>
<td>LNCaP coengrafted with bone marrow cells</td>
<td>Mutant</td>
<td>No</td>
<td>58</td>
</tr>
<tr>
<td>CWR22Rv1</td>
<td>Human prostate cancer</td>
<td>Mutant</td>
<td>No</td>
<td>42</td>
</tr>
<tr>
<td>RWPE-1</td>
<td>Human prostate epithelium transformed by HPV-18</td>
<td>Wild type</td>
<td>Yes</td>
<td>41</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryo kidney transformed by adenovirus 5</td>
<td>Null</td>
<td>No</td>
<td>59</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Human breast cancer</td>
<td>Positive</td>
<td>Yes</td>
<td>60, 61</td>
</tr>
<tr>
<td>T47D</td>
<td>Human breast cancer</td>
<td>Positive</td>
<td>No</td>
<td>62</td>
</tr>
</tbody>
</table>

Cytotoxicity Assays and Flow Cytometry

Typically, cell viability was assessed with a trypan blue exclusion assay as described in our previous publication (33). Apoptotic cell death was determined using an annexin V-FITC Apoptosis Detection kit (BD PharMingen, San Diego, CA) according to the manufacturer’s manual. Briefly, cells were harvested and washed with ice-cold PBS and then suspended in annexin V binding buffer. Then, cells were stained for 15 minutes at room temperature in the dark and analyzed on a FACSCalibur flow cytometer using CELL-Quest software. For clonogenic survival assay, 10^5 cells were seeded in a 35 mm dish and transfected with the siRNAs as indicated in the figure legend. The media were changed every 3 days and the cultures were observed daily for colony formation. On day 7, the cultures were washed with PBS, fixed, and stained as previously described (36). The colonies were counted under an inverted microscope.

mRNA Expression Analysis and Reverse Transcription-PCR

Total RNA was prepared using Trizol reagent (Invitrogen). To assess mRNA expression, a semiquantitative reverse transcription-PCR (RT-PCR) method was used as described previously (35). RT-PCR was done using a RETROscript kit (Ambion) per manufacturer’s instructions. The primers and PCR conditions were described as follows: for human AR gene (forward 5'-ctgtggctccgaaactacac-3'; backward 5'-ggacttgtgcatgcggtactca-3'; adapted from ref. 6); human PSA gene (forward 5'-gtgacttcagccagcagct-3'; backward 5'-cacagacacccactctc-3'; ref. 37); and human bcl-xl gene (forward 5'-catggcagcatgtaaaacag-3'; backward 5'-gcatttgcctatagatc-3'; ref. 38). 28S ribozyrne RNA (forward 5'-gttaccactaatgaaacaggtg-3'; backward 5'-gattgtcgagctgctgct-3') was used as an internal control. The primers were synthesized by IDT. The amplification profile was as follows: 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute running in a total of in 25 cycles. After 25 amplification cycles, the expected PCR products were size fractionated onto a 2% agarose gel and stained with ethidium bromide.

Mitochondrial Membrane Potential and Caspase Activity

The siRNA-transfected cells were incubated in the presence of JC-1, which was added to the culture medium at a final concentration of 0.3 μM/mL for 15 minutes at 37°C. Thereafter, the cells were analyzed under a fluorescent microscope. The caspase activity was measured using an Apo-ONE Homogeneous Caspase-3/7 Assay kit obtained from Promega (Madison, WI) per the manufacturer’s manual. Briefly, the cells were washed in ice-cold PBS and then suspended in the assay buffer containing the substrate rhodamine 110 (Z-DEVD-R110) provided by the supplier. The amount of fluorescent product generated is measured at 480/520 nm (wavelength) using a Fluoscan fluorescent reader as described previously (32, 34).

Statistical Analysis

All experiments were repeated twice or thrice. Western blot results are presented from a representative experiment. The mean and SD from two experiments for cell viability are shown. The number of viable/dying cells or cell colonies in the control group or the initial time point was assigned a relative value of 100%. The significant differences between groups were analyzed using the SPSS computer software (SPSS, Inc., Chicago, IL).

Results

Knocking Down AR Expression via RNA Interference Approach in Prostate Cancer Cells

Because the AR has been shown to play a critical role in hormone-refractory progression of prostate cancer (6–17), targeting the AR gene by reducing its translation or blocking its function via antisense approach has emerged as a novel strategy for prostate cancer therapy (13–15). Recently, RNA interference has been shown to be a better strategy in blocking gene expression in cultured cells or animal model (29–31). To explore the feasibility of the RNAi technique in knocking down AR expression in prostate...
cancer cells that harbor the AR gene, we designed and synthesized a panel of siRNAs against human AR gene. Two relatively potent siRNAs (AR siRNA 8, 5'-AAGAAG-GCCAGUUGUAUGGAC-3'; AR siRNA 31, 5'-AAGAGCCUUCUACCACUCAC-3') were identified in knocking down AR expression in the initial experiments when compared with others. The AR knockdown effect was further confirmed by checking the mRNA level followed by Western blot. A well-known androgen target prostate-specific antigen was also down-regulated as determined by a RT-PCR assay. This knocking down effect was achieved as a sequence-specific event because a negative control siRNA with a scrambled sequence had no effect on AR protein or prostate-specific antigen mRNA level (Fig. 1A). Both AR siRNAs 8 and 31 significantly knocked down AR expression at a final concentration of 1.0 to 10 nmol/L in culture media after 4 days in LNCaP cells that harbor an endogenous mutant AR gene, as well as in PC-3/AR cells that were reconstituted with an exogenous wild-type AR gene (Fig. 1B). Moreover, the knocking down effect of the AR protein was further verified using an immunofluorescent staining approach where LAPC4 cells, which harbor an endogenous wild-type AR gene, were used (Fig. 1C). These results show that the RNAi machinery is functional in prostate cancer cells, which is consistent with two recent reports (8, 39), and can be activated by a siRNA duplex.

siRNA-Mediated AR Silencing Leads to Dramatic Cell Death

It is shown that the AR is a key factor for cell proliferation in vitro (13, 14) or tumor growth in vivo (15) in prostate cancer. Consistent with two recent reports showing a reduced cell proliferation after AR protein was knocked down via the RNAi approach (8, 39), we also found that cell growth was largely reduced after transfection of LAPC-4 cells with either AR siRNA 8 or a pooled AR siRNA mixture (Fig. 2A). However, the difference was that a massive cell death was observed if the cells were monitored for >4 days after siRNA transfection. To test if the cell death response is due to siRNA-mediated AR silencing, we did a time course experiment in LNCaP (hormone-sensitive) and C4-2 (hormone-refractory) cells. The cells were transfected with AR siRNA 8 or a scrambled negative siRNA in 2% charcoal-stripped FBS. The relative survival rate of the cells was determined every 2 days using a trypan blue exclusion assay. Transfection of the cells with the AR siRNA duplexes resulted in a significant cell death in which LNCaP cells (Fig. 2B) showed a quicker response compared with C4-2 cells (Fig. 2C). In contrast, the negative control siRNA did not cause cell death. These data suggest that the AR siRNA induces cell death regardless of hormone sensitivity, although C4-2 cells showed a delayed response compared with LNCaP cells.

Next, we asked if the AR siRNA-induced cell death was simply due to a cellular nonspecific response to the double-stranded siRNA (i.e., IFN response; ref. 40) or those degraded AR mRNA produced by the RNAi machinery. The experiments were conducted using PC-3/AR, PC-3/Neo (empty vector control subline), and LNCaP-Rf (hormone-refractory, ref. 13) cell lines. As shown in Fig. 2D, either AR siRNA 8 or 31 significantly reduced the survival rate for >95% in LNCaP-Rf cells compared with the control siRNA. In contrast, the cell survival rate...
was not affected in either PC-3/AR or PC-3/Neo cells after the siRNA transfection. These data suggest that the AR siRNA–induced cell death in the native AR-harboring cells is not a nonspecific cellular response to the double-stranded siRNA or siRNA-mediated AR mRNA degradation but due to a disruption of the survival machinery that depends on the AR. In the AR-null cells, like PC-3/Neo or PC-3/AR cells where an exogenous AR gene is expressed, the survival machinery of the cells might not depend on the AR.

**AR siRNA–Induced Cell Death Occurs Specifically in Prostate-Derived Cells**

In addition to those commonly used prostate cancer cells as mentioned above, we also tested the cell death response to the AR siRNA in two more prostate epithelial cell lines (RWPE-1 and CWR22Rv1) and breast cancer cell lines (MCF-7 and T47D) to verify the specificity of AR siRNA–induced cell death. The RWPE 1 is a nontumorigenic prostate epithelial cell line (41), whereas the CWR22Rv1 is a hormone-refractory prostate cancer cell derived from CWR22 xenograft (42). Although the CWR22Rv1 cells, like C4-2 cells, showed a delayed response to AR siRNA–induced cell death, the nontumorigenic RWPE-1 cell line showed a rapid death response even faster than LAPC-4 (Fig. 3A) and LNCaP cells (Fig. 2B). However, the two breast cell lines did not show any cell death response to the AR siRNA although they are also harboring an endogenous AR (data not shown). A selected data for AR siRNA–induced AR protein knockdown in CWR22Rv1 and LAPC-4 cells was shown in Fig. 3B.

To visualize the specificity of the AR siRNA–induced cell death, we labeled AR siRNA 31 with a fluorescent dye (Cy3) and then transfected into LNCaP cells. Cells were maintained in 2% charcoal-stripped FBS and cell death was monitored daily under a fluorescent microscope. As shown in Fig. 4, the Cy3-labeled siRNA was seen in a large population of the cells, indicating a successful transfection. Most interestingly, only the dying cells (round and detached from the plastic) showed a positive Cy3 labeling (Fig. 4, black arrow); however, living cells (spreading and attached cells) showed no Cy3 labeling (Fig. 4, white arrow). These data show the specific effect of the AR siRNA–induced cell death only on the transfected cells.

**Mitochondrial Apoptotic Mechanism Is Involved in AR siRNA–Induced Cell Death**

It has been shown that androgen ablation or antiandrogens induces apoptotic cell death in prostate epithelium and
prostate cancer cells (3). To determine if AR siRNA–induced cell death is an apoptotic response, we first detected the change of the membrane phospholipid phosphatidylserine, which is translocated from the inner to the outer leaflet of the plasma membrane during the earlier phase of apoptosis (43). As shown in Fig. 5A, transfection of the cells with the AR siRNAs induced significant phosphatidylserine translocation, whereas the control siRNA had no effect.

Because loss of mitochondrial transmembrane potential ($\Delta \psi_{m}$) is considered to be one of the central events in apoptotic death that leads to incapacitation of the mitochondria, release of cytochrome $c$, and activation of the caspase pathway, we tested the integrity of this signaling event using the fluorescent dye JC-1 as described elsewhere (44). Upon entering the mitochondrial negative transmembrane potential in healthy cells, JC-1 forms red fluorescent aggregates. When the transmembrane potential is low, as in many cells undergoing apoptosis, JC-1 exists as a monomer and produces green fluorescence. Consistent with this notion, green fluorescence was observed in dying cells after being transfected with AR siRNA 8 (as pointed by arrows in Fig. 5B, c and d), whereas living cells remained normal membrane potential (red fluorescence as pointed with arrowhead in Fig. 5B).

The presence of cytochrome $c$ in the cytosol is a critical event required for the correct assembly of the apoptosome, subsequent activation of the executioner caspases, and induction of cell death (45). To evaluate the release of cytochrome $c$, cytosolic fraction of the cellular protein was collected 6 days after siRNA transfection. As shown in Fig. 6A, in parallel with the AR knocking down, cytochrome $c$ was detected in the cytosolic fraction when AR siRNA 8 was transfected into cells. Meanwhile, the apoptosis hallmarker poly(ADP-ribose) polymerase cleavage fragment was also detected. Finally, the proteolytic processing of inactive procaspases, the essential component of the death pathway in many cells (21), and their catalytic activity were also analyzed. As shown in Fig. 6B, transfection with AR siRNA 31 into LNCaP cells induced significant reduction of procaspase-3, procaspase-6, and DFF45 (evidence for proteolytic activation or cleavage). Similar results were also seen when LAPC-4 or C4-2 cells were used (data not shown). Consistently, the catalytic activity of caspase 3/7 was significantly increased when AR siRNA 31 was used compared with negative control siRNA (Fig. 6C). Thus, these data clearly showed that the mitochondrial apoptotic mechanism is activated by the AR siRNAs.

**Antiapoptotic Protein Bcl-xL Is Involved in AR-Mediated Cell Survival**

Having shown the mitochondrial involvement in AR siRNA–induced cell death, we next focused on the Bcl-2 family members because they are the major regulators of mitochondrial function in the aspect of apoptosis by facilitating or inhibiting cytochrome $c$ release to cytosol.

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**Figure 3.** AR siRNA specifically induces cell death in other prostate-derived cells. A, three prostate cell lines (RWPE-1, LAPC-4, and CWR22Rv1) were transfected with AR siRNA 8 at 10 nmol/L in the culture medium supplied with 2% charcoal-stripped FBS, and cell survival rate was determined 7 days later by trypan blue exclusion assay. B, cells harvested from the experiments described in (A) were lysed for Western blot analysis to determine the protein levels of the AR. Actin blot served as loading control.

**Figure 4.** Visualization of the Cy3-labeled AR siRNA–induced cell death. LNCaP cells were seeded in six-well plates overnight and then transfected with Cy3-labeled siRNAs (10 nmol/L in the medium) as indicated. Cell death was monitored daily. Pictures were taken at days 1 and 4 after transfection. The Cy3-labeled siRNAs are seen as white dots in Cy3 (b, d, f, and h). In (g) and (h), white arrows indicate several living cells without the Cy3 labeling (negative transfection), whereas black arrows indicate a cluster of dying cells (round and detached) with strong Cy3 labeling (positive transfection).
and subsequent assembly of an active apoptosome (22). These functions are promoted by the proapoptotic Bax or Bak and are inhibited by the antiapoptotic Bcl-2 and Bcl-xL. We determined whether protein expression of these Bcl-2 family members is altered after the AR siRNA transfection. Interestingly, we found that the protein level of the antiapoptotic member Bcl-xL dramatically decreased in the AR siRNA 8–transfected cells compared with the controls, whereas another antiapoptotic member, Bcl-2, and the proapoptotic members, Bax and Bak, remained unchanged (Fig. 7A). To better illustrate the relationship of Bcl-xL reduction with AR silencing, we conducted a time course experiment (Fig. 7B). The protein levels of Bcl-xL decreased in a time-dependent manner following the AR siRNA transfection; however, Bax protein remained consistent during the time course. These data indicate that AR silencing results in Bcl-xL reduction that might lead to an imbalance between the proapoptotic and antiapoptotic members of the Bcl-2 family that, in turn, triggers apoptosis.

To shed light onto the mechanistic basis underlying the response of Bcl-xL reduction to AR silencing, we also examined Bcl-xL expression at the mRNA level by RT-PCR assay. As shown in Fig. 7C, the Bcl-xL mRNA level decreased significantly after AR siRNA 8 transfection compared with the controls, indicating that the reduction of Bcl-xL protein after AR silencing is via a transcriptional mechanism.

Figure 5. AR siRNA induces apoptotic cell death. A, after transfection with the siRNA duplexes (10 nmol/L in the medium) as indicated for 4 d, LNCaP cells were harvested and the change of the membrane phospholipid phosphatidylserine was determined using fluorescence-activated cell sorting for FITC-labeled cells as described in the text. Data are from two different experiments. B, following transfection with the siRNA duplexes (10 nmol/L in the medium) for 5 d, LNCaP cells were incubated with JC-1 (0.3 μg/mL) for 15 min at 37°C. Pictures were taken under a fluorescent microscope (magnitude ×200).

Figure 6. AR siRNA induces cytochrome c release, caspase activation, and cleavage of DFF45 and poly(ADP-ribose) polymerase. A and B, after 7 d of transfection with the siRNAs as indicated, LNCaP cells were harvested and the cytosolic occurrence of cytochrome c, proteolytic process of caspase-3 and caspase-6, and DFF45 and poly(ADP-ribose) polymerase cleavage were determined by Western blot. C, after 7 d of transfection with the siRNAs as indicated, LNCaP cells were washed with ice-cold PBS and then harvested. Caspase activity was measured as described in the text. Columns, mean value of relative activity from three independent experiments.
AR siRNA–Induced Apoptosis Was Partially Inhibited by Ectopic Bcl-xL Expression

In view of the antiapoptotic feature of Bcl-xL protein, we hypothesized that the AR promotes cellular survival by upregulating the bcl-x gene expression through a transcriptional mechanism in prostate cancer cells. Therefore, Bcl-xL expression will decrease if the AR is knocked down, which subsequently results in apoptosis due to an imbalance between the proapoptotic and antiapoptotic members of the Bcl-2 family. Thus, we wondered if an enforced Bcl-xL expression will protect cells from apoptosis while AR is silenced. To assess the protection effect of Bcl-xL protein, a stable LNCaP subline overexpressing human Bcl-xL protein controlled by a cytomegalovirus promoter (LNCaP/Bcl-xL) or a control subline with an empty vector (LNCaP/ puromycin) were established. Consistent with the results obtained from the parental cells (Fig. 7A), exposure of those LNCaP subline cells to AR siRNA 8 resulted in a decrease of endogenous but not exogenous Bcl-xL protein (Fig. 8A, lane 1 versus lane 2). Most significantly, as expected, enforced Bcl-xL expression partially inhibited cell death induced by AR siRNA transfection in LNCaP/Bcl-xL cells compared with the controls (Fig. 8A, bottom). These data showed that Bcl-xL is involved in AR-mediated survival of prostate cancer, and the reduction of Bcl-xL expression after AR silencing represents a mechanism for the AR siRNA–induced apoptosis.

In addition, while establishing a subclone for stable Bcl-xL expression in LNCaP cells, an unexpected clone (LNCaP subclone 11 (LN11)) was obtained, in which Bcl-xL expression was dramatically reduced for unknown reason, as confirmed by RT-PCR and Western blotting (Fig. 8B, top and middle). By taking advantage of this particular clone of LN11 subline cells, we further tested the involvement of Bcl-xL in AR-mediated survival. Exposing LN11 subline cells to AR siRNA 8 resulted in a significant increase in AR siRNA–mediated cell death compared with the parental LNCaP cells and untransfected controls (Fig. 8B, bottom), although the LN11 subline did not show a profound cell death without AR silencing. These data indicate that loss of Bcl-xL expression enhances AR siRNA–induced cell death, and multiple downstream factors, besides Bcl-xL, are mediating AR survival signal.

Discussion

In this report, we identified two siRNA duplexes that induce a strong AR silencing in prostate cancer cells. Most importantly, we found that siRNA-mediated AR silencing subsequently leads to massive cell death through a mitochondrial apoptotic pathway. AR siRNA–induced apoptosis only occurs in prostate cancer cells that harbor an endogenous AR regardless of their androgen sensitivity. Further analyses showed that Bcl-xL expression is transcriptionally dependent on the AR in prostate cancer cells, and siRNA-mediated AR silencing results in a reduction of Bcl-xL expression that accounts partially for the apoptotic response because enforced Bcl-xL expression inhibited cell death after AR silencing. To the authors’ knowledge, this is the first report showing AR involvement in Bcl-xL expression and apoptotic response to siRNA-mediated AR silencing in prostate cancer.
Our results are somewhat different from other approach-induced AR blockage as mentioned above (8, 13–15, 39), in which only cell arrest or reduced tumor growth, but no cell death, were reported. The plausible reason might be the difference in the extent of AR blockage or protein knockdown. For example, the AR-specific antibody might not totally block the AR function as used in a previous report (13) because the AR protein still exists in the cell. It is believed that the RNAi approach is more potent than the ribozyme (13) or antisense approach in terms of gene silencing (14, 15); therefore, our RNAi approach might have induced a more efficient knocking down of the AR protein than the former approach of AR ribozyme or antisense oligonucleotides. In addition, current experiences in the field of RNAi technology showed that the siRNAs targeted to different regions of a gene transcript may not function equally (31), which may be responsible for the different findings between our results and others (8, 39). Finally, the strategies in the experimental condition used between ours and other groups (8, 39) might also account for the different outcome.

It has been shown that androgen or other factors as critical survival stimuli play an important role via the AR in prostate cancer progression. Although AR-dependent functional repression of FKHR and related FOXO forkhead proteins was reported as a possible pathway (18), the survival pathway of AR-dependent mechanism is not clear. PI3K-Akt is a major cellular survival factor that is negatively regulated by the PTEN phosphatase (46). In LNCaP cells, Akt is constitutively active due to PTEN mutational inactivation (47), whereas LAPC-4 cells maintain a wild-type PTEN (48). Here, we observed that AR siRNA induced cell death in both of the cell lines, suggesting that the AR-dependent survival pathway is via an AKT-independent mechanism, which was also proposed by previous reports (49, 50). In addition, we observed the apoptotic response from those native AR-harboring cells (RWPE-1, LAPC-4, LNCaP, CWR22Rv1, and C4-2), but not from the AR-null PC-3 or its subline PC-3/AR cells, which was reconstituted with an exogenous AR gene. These findings indicate that the AR is a critical survival factor for prostate epithelium-derived cells and remains as an important survival factor even in those hormone-refractory cancer cells, although they might have developed additional survival mechanism. However, the AR-null prostate cancer cells already escaped from the AR-regulated survival control.

The Bcl-2 family proteins reside immediately upstream of mitochondria and function as either death antagonists.
or agonists. The ratio of death antagonists to agonists determines how a cell responds to an apoptotic signal. Like Bcl-2, Bcl-xL is another major apoptotic antagonist and its expression is mainly regulated through transcriptional mechanisms (21, 22). The bcl-x promoter contains consensus motifs for a number of transcription factors, including Sp1, activator protein-1, Oct-1, Ets, Rel/NF-κB, signal transducers and activators of transcription (STAT), and GATA-1, in which three transcription factor families, STATs, Rel/NF-κB, and Ets family, have been shown to play an important role in the regulation of the bcl-x gene expression (26, 27). Recently, other steroid hormone receptors, including receptors for glucocorticoid and progesterone, have been reported to bind to the mouse bcl-x promoter (51, 52). In this report, our data suggest that the AR is involved in the transcriptional regulation of bcl-x gene expression, although the underlying mechanism is under further investigation by our group.

Recently, siRNA-mediated IFN response has emerged as a big concern regarding the use of siRNA in mammalian cells (40, 53). In our system, we also observed the similar response in which the transcriptionally made siRNAs induced more significant IFN response than the chemically synthesized ones. However, similar responses were observed in all of those prostate cancer cells used in our study, indicating that the apoptotic effect of AR siRNA in the AR-harboring cells is independent of the IFN-related effect. Moreover, it was reported that only tumor necrosis factor-α, but not IFN, down-regulates Bcl-xL expression (54), suggesting that the reduction of Bcl-xL protein is not due to the siRNA-triggered IFN response.

In conclusion, our results showed for the first time that knocking down the AR protein by a siRNA duplex induces apoptosis in native AR-positive prostate cells regardless their hormone sensitivity. The apoptotic response induced by the AR siRNAs is partially due to reduction of Bcl-xL expression because enforced Bcl-xL expression inhibits AR siRNA-induced cell death. Currently, the underlying mechanism for AR-mediated up-regulation of the bcl-x gene is under further investigation. The siRNA-mediated AR silencing may be implicated as a novel approach in the future for curing the hormone-refractory prostate cancers that are currently considered as a condition with no cure.

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5Unpublished observation.

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