Signaling Pathway Activated during Apoptosis of the Prostate Cancer Cell Line LNCaP: Overexpression of Caspase-7 as a New Gene Therapy Strategy for Prostate Cancer

Marco Marcelli,2 Glenn R. Cunningham, Margaret Walkup, Zening He, Lydia Sturgis, Carolina Kagan, Roberta Mannucci, Ildo Nicoletti, Babie Teng, and Larry Denner

Deans of Medicine [M. M., M. G. R. C., M. W., Z. H.] and Cell Biology [M. M., G. R. C.], Veterans Administration Medical Center and Baylor College of Medicine, Houston, Texas 77030; Institute of Internal Medicine and Oncologic Sciences, Perugia University Medical School, I-06100 Perugia, Italy [R. M., I. N.]; Institute of Molecular Medicine, University of Texas, Houston Health Science Center, Houston, Texas 77030 [B. T.]; and Department of Cell Biology, Texas Biotechnology Corporation, Houston, Texas 77030 [L. S., C. K. R. L. D.]

ABSTRACT

We studied the molecular mechanisms of apoptosis in the prostate cancer cell line LNCaP and whether overexpression of caspase activity could force this cell line to undergo apoptosis. The inhibitor of phospho-
evalonate decarboxylase, sodium phenylacetate, and the protein kinase inhibitor staurosporine induced (a) release of cytochrome c from the mitochondria to the cytosol; (b) reduction in mitochondrial transmem-
brane potential; (c) proteolytic processing of caspase-3 and -7 but not -2; (d) cleavage of the DEVD substrate and the death substrates poly(ADP-ribose) polymerase and DNA fragmentation factor; and (e) apoptosis. The pan-specific inhibitor of caspase activation, N-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (z-DEVD-FMK) prevented all of these events except release of mitochondrial cytochrome c into the cytosol. None of these apoptotic signaling events were elicited by staurosporine or sodium phenylacetate treatment of LNCaP-Bcl-2 cells that overexpress the oncoprotein Bcl-2. Because caspase-7 is activated in every model of apoptosis that we have characterized thus far, we wished to learn whether overexpression of this protease could directly cause apoptosis of LNCaP cells. By using a replication-defective adenovirus, overexpression of caspase-7 protein in both LNCaP and LNCaP-Bcl-2 cells was accompanied by induction of cleavage of the DEVD substrate and TUNEL. These studies have demonstrated that caspase-7 and -3 are critical mediators of apoptosis in LNCaP cells. Caspase-7 was proteolytically activated in every model of apoptosis that we have developed, and the overexpression of it induced apoptosis of LNCaP and LNCaP-Bcl-2 cells. Thus, adenovirally-mediated transfer of caspase-7 may offer a new effective approach for the treatment of prostate cancer.

INTRODUCTION

Prostate cancer remains the second leading cause of cancer deaths in American men. The number of patients who died from this disease in 1997 accounts for 14% of all cancer deaths in males (1). Radical prostatectomy is the main curative treatment for men with organ-confined disease. Most men with non-organ-confined disease will undergo palliation with radiation or androgen ablation (2). Androgen ablation successfully shrinks primary and metastatic lesions by inducing apoptosis or PCD3 of androgen-dependent prostate cancer cells (3). Unfortunately, non-organ-confined prostate cancer is a heterogeneous lesion and, at the time of diagnosis, contains foci of both androgen-dependent and -independent cells (4). Androgen-independent cells escape apoptosis induced by androgen ablation (5) and by many cytotoxic drugs. They continue to proliferate and metastasize despite profound changes in the surrounding hormonal milieu and represent the most direct threat to patient survival.

Despite its cryptic nature, the apoptotic machinery of prostate cancer cells is still in place (6) and can be activated under certain circumstances (7). Therefore, we have hypothesized that the apoptotic machinery of prostate cancer cells can be activated for tumoricidal purposes and that an undesirable cell (e.g., an androgen-independent prostate cancer cell) can be forced to commit PCD. LNCaP cells have an androgen receptor, and their growth is increased by androgen. However, because they do not undergo apoptosis after androgen withdrawal, these cells can be used as an in vitro model to study strategies for treating prostate cancers that are resistant to androgen ablation. We previously described experiments in which the cholesterol-lowering medication lovastatin induced apoptosis of LNCaP cells (7) and demonstrated the involvement of the caspase family of proteases in mediating apoptosis in this model. Caspase-7 was identified as one of the possible mediators of lovastatin-induced apoptosis.

The goals of the current study were several (a) to identify the caspase(s) activated in other models of apoptosis; (b) to determine the molecular steps of the apoptotic pathway that were induced upstream and downstream of caspase activation; (c) to study the molecular basis of resistance to apoptosis in a LNCaP cell line stably transfected with the oncoprotein Bcl-2; and (d) to determine whether overexpression of a caspase activated during chemically induced PCD of LNCaP cells would cause apoptosis of this cell line and of LNCaP-Bcl-2 cells in the absence of apoptogenic stimuli. Here we present evidence furthering our understanding of the molecular events associated with apoptosis of LNCaP cells and demonstrate that the manipulation of caspase-7 is a potential gene therapy strategy to force prostate cancer cells to undergo PCD.

MATERIALS AND METHODS

Materials. NPA was provided by Dr. W. Perkins (Elan Pharmaceutical Research Corporation, Gainesville, GA). STS was purchased from Sigma Chemical Co. (St. Louis, MO). Chemicals and protease inhibitors were purchased from Sigma and Boehringer (Indianapolis, IN). Fetal bovine serum and tissue culture media were from Life Technologies, Inc. (Gaithersburg, MD). The caspase inhibitors, z-DEVD-FMK and z-DEVDFMK, and the fluorogenic substrate, z-DEVDF-AFC, were from Enzyme System (Dublin, CA). The en-

Received 7/29/98; accepted 11/11/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by a Merit Review Grant from the Veterans Administration, by a Pilot Project Grant from the Baylor Specialized Programs of Research Excellence on Prostate Cancer, by a Grant from the Pfeiffer Foundation (to M. M.), and by a Grant from Associazione Italiana Picerca sul Cancro (AIRC) and Centro Universitario Ricerca Oncologica (CIBIO) (to I. N.).

2 To whom requests for reprints should be addressed, at Department of Medicine, Baylor College of Medicine and Veterans Administration Medical Center, 200 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 794-7945; Fax: (713) 794-7714; E-mail: marcelli@bcm.tmc.edu.

3 The abbreviations used are: PCD, programmed cell death; Δψm, mitochondrial transmembrane potential; NPA, sodium phenylacetate; STS, staurosporine; z-VDAD-FMK, N-benzyloxycarbonyl-Val-Ala-AspOMe-fluoromethylketone; z-DEVD-FMK, N-benzyloxycarbonyl-AspOMe-GluOMe-ValOMe-fluoromethylketone; AIF, apoptosis-inducing factor; PARP, poly(ADP-ribose) polymerase; DFF, DNA fragmentation factor; ICAD, inhibitor of caspase-activated DNase.

382
hanced chemiluminescence detection reagents were from Amersham (Arlington Heights, IL). JC-1 was from Molecular Probes (Eugene, OR).

**Cell Culture.** LNCaP (obtained from the American Type Culture Collection) were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. LNCaP-Bcl-2 (Ref. 8; gifts of Dr. R. Buttyan, Columbia University, New York, NY) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, and 400 µg/ml of Genetcyte (Life Technologies).

STS and NPA dissolved in DMSO (Sigma) at concentrations 1000-fold higher than the working concentrations were administered for various lengths of time and doses (see “Results”). Controls received DMSO at a concentration equal to that of the treated cells.

**Apoptosis Assay.** Apoptosis was determined by DNA laddering, TUNEL, and morphological criteria as described previously (7).

**Confocal Microscopy.** LNCaP and LNCaP-Bcl-2 cells were grown on 25-mm glass coverslips in RPMI 1640 and 10% FCS. For mitochondrial analysis, cells were loaded with 5 µM JC-1 in RPMI 1640 for 10 min at 37°C. Cells were then washed twice with PBS and transferred to an Attofluor micro chamber (Molecular Probes) for confocal analysis on a MCR-1024 Bio-Rad apparatus. A representative microscopic field was chosen and a baseline image was recorded. The 488-nm laser line was used for excitation, and red and green emissions of the sample were simultaneously recorded. The microscopic field, laser potency, and photomultiplier gain were kept constant for the whole experiment. Images were processed with the LaserSharp 1024 (Bio-Rad) and reanalyzed with Optitap-Pr softwares (Graftek Imaging, Austin, TX) on a PowerPC personal computer (Macintosh 8100, Apple, Cupertino, CA). STS (4 µM) was then added and images were registered after 10, 20, 30, 40, and 60 min. For the 4-h experiments, cells were grown on coverslips as described above and incubated with 4 µM STS for 4 h. When the pan caspase inhibitor was used, cells were pretreated for 1 h with 100 µM z-VAD-FMK. After a 4-h incubation, cells were washed with 5 µM JC-1 for 1 min at 37°C in culture medium. Cells were then washed twice with PBS, the coverslip was mounted onto the Attofluor chamber, and confocal analysis was performed as described above.

**Western Analysis.** The techniques and the antibodies for the immunodetection of PARP, caspase-3, and caspase-7 have been described previously (7). For the immunodetection of caspase-2 (Transduction Laboratories, Lexington, KY) and DFF (antibody provided by Dr. Xiaodong Wang, University of Texas Southwestern Medical Center, Dallas, TX), we used the same technique used for caspase-3 and -7. Cytosolic extracts free of mitochondria were prepared for the immunodetection of cytochrome c as described by Bossy-Wetzel et al. (9). Briefly, LNCaP cells grown in 10-cm dishes were collected by microcentrifugation at 1500 rpm at 4°C for 2 min and washed twice in ice-cold PBS. The cell pellet was resuspended in 200 µl of extraction buffer [220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 2 mM MgCl₂, 1 mM DTT, and protease inhibitors (complete™, Boehringer, Indianapolis IN)]. After 30 min at 4°C, cells were homogenized with a glass dounce and a B pestle (40 strokes). The homogenates were spun at 13,000 rpm for 15 min, and the supernatants were stored at −70°C. Cytosolic protein extracts (50 µg) were then sized in 17% SDS-polyacrylamide gel, blotted, and analyzed using monoclonal antibody 7H8.2C12 (PharMingen, San Diego, CA). Immunoreactive bands were visualized using the enhanced chemiluminescence detection reagents from Amersham Corp. (Arlington Heights, IL).

**Assay of DEVDDase Activity.** The catalytic activity of the caspases was measured as described previously (7) using a fluorometric assay monitoring the cleavage of the fluorogenic tetrapeptide z-DEVD-AFC.

**Inhibition of Caspase Activity.** Inhibition of caspases with DEVDD cleavage activity was achieved in vivo using the inhibitors z-DEVDD-FMK and z-DEVDD-FMK administered at 100 µM 1 h before the addition of STS (4 µM). Twelve h after the addition of STS, cells were harvested and analyzed by TUNEL, Western analysis, or DNA electrophoresis.

**Preparation of Adenovirus.** A caspase-7 cDNA encompassing amino acids 1–303 (10) of caspase-7 was obtained by reverse-transcription PCR of LNCaP mRNA. The product of the amplification was subcloned in the BamHI and XhoI sites of plasmid pCDNA-3 (Invitrogen, Carlsbad CA). The absence of mutations in the resulting construct was evaluated by sequence analysis. Replication-defective recombinant adenoviruses were produced as reported previously (11). Briefly, the caspase-7 cDNA was subcloned in vector pAVS6 (12) to obtain the shuttle plasmid pAVS6–C7. pAVS6 has a lBluescript (Stratagene) backbone and contains sequences from the left end of the adenovirus 5 genome lacking the early transcription region-1 (E1) that is necessary for replication. In this plasmid, the gene of interest is subcloned in a polynucleoty site located downstream from the RSV promoter and upstream to the SV40 early polyadenylation signal. pAVS6–C7 was cotransfected with pMJ17 (13), which contains a full-length adenoviral genome, in low-passage 293 cells (14). This cell line is stably transfected with the SV40 virus and provides E1 functions in trans (Microbix Biosystem, Toronto, Canada). E1-defective recombinant adenovirus (Av-C7) was produced by homologous recombination between pAVS6–C7 and pMJ17 in 293 cells. Two weeks after transfection, infectious recombinant adenoviral plaques were picked, expanded, and screened for caspase-7 sequences by PCR. Adenoviral vectors that contained C7 were purified one more time by plaque assay on 293 cells, and recombinant Av-C7 was confirmed by sequencing and restriction-enzyme mapping. Recombinant adenovirus Av-lacZ, which contains the lacZ cDNA under the control of the RSV promoter, was produced as described previously (11). Large-scale production of high-titer recombinant adenovirus was performed by growing 293 cells on improved Eagle’s MEM supplemented with 10% fetal bovine serum, 2 mM t-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, and 1% Fungizone (Life Technologies). The virus was purified twice using cesium chloride density gradient centrifugation. The viral vector was then dialyzed for ≥8 h at 4°C against a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, and 10% glycerol and was stored at −80°C.

**Infection of LNCaP Cells with Viral Constructs.** In preliminary experiments, we titrated the concentration of Av-C7 that achieved apoptosis of LNCaP cells in the most effective and specific way. Experiments were performed in which 4 × 10⁶ LNCaP cells (seeded in 6-well plates) were infected with 10⁶–10¹³ viral particles of Av-C7, the control virus Av-lacZ, or vehicle alone. At the time points of 24, 48, 72, and 96 h, cells were harvested and analyzed for TUNEL. Because the apoptogenic activity of Ad-C7 was most effective at the titer of 10¹⁰, LNCaP and LNCaP-Bcl-2 cells were treated with 10¹⁰ viral particles of Av-C7 or Av-lacZ for 0–96 h. At the time points of 24, 48, 72, and 96 h, cells were harvested and analyzed for caspase-7 expression and processing, TUNEL, and DEVD-ase activity.

**RESULTS**

**Apoptosis of LNCaP Cells.** To determine whether NPA and STS induce the apoptosis of LNCaP cells, LNCaP cells were incubated with increasing concentrations of NPA (0.1–10 mM for 96 h) and STS (4 mM–40 µM for 24 h; data not shown). The minimal concentration that induced reproducible apoptosis assessed by DNA laddering was used for subsequent experiments. Fig. 1 shows the kinetics of STS (1A) and NPA (1B) induced apoptosis. Treatment with NPA (10 mM) was associated with induction of apoptosis after 48 h of treatment. STS (4 µM) was considerably more rapid and induced apoptosis by 6 h. After 24 h of treatment with STS, 87 ± 4% of the cells were TUNEL-positive, in comparison with 4.2 ± 0.4% of the cells receiving vehicle alone (Table 1). After 72 h of treatment with NPA, 80 ± 6% of the cells were TUNEL-positive, in comparison with 6 ± 0.98% of the cells receiving vehicle (Table 1).

To evaluate mitochondrial involvement, we induced apoptosis with STS and used confocal microscopy of LNCaP cells stained with the fluorescent cyanine dye JC-1 (Fig. 2), which is specifically taken up by the mitochondria. When the transmembrane potential (∆ψₘ) of the mitochondria is high, like it is in a normal cell, JC-1 forms red fluorescent dimers. However, when the ∆ψₘ is low, as in mitochondria of cells undergoing apoptosis, the red fluorescence disappears. After treatment with STS for 1 (data not shown) or 4 h (Fig. 2, A and B), the red mitochondrial fluorescence disappeared, which indicates a decrease in ∆ψₘ concomitant with the induction of apoptosis. Other morphological changes of LNCaP cells undergoing apoptosis included ballooning of the mitochondria (data not shown).

**Apoptosis of LNCaP-Bcl-2 Cells.** The ability of STS (4 µM) to induce apoptosis of LNCaP-Bcl-2 cells was also determined. DNA laddering (Fig. 1C) and TUNEL (2.3 ± 0.2% versus 3.2 ± 0.1% cells were TUNEL-positive after 24 h in the STS and control group,
Although the experiments shown in Fig. 4 suggest that caspase-7 was cleaved before caspase-3 (the $M_r$ 20,000 active subunit of caspase-7 appeared after 1 h of STS and 24 h of NPA treatment), this may simply be related to differences in the ability of the antibodies that were used to detect the active subunits or in the stability of the active subunits of caspase-3 and -7.

Because LNCaP-Bcl-2 cells failed to undergo apoptosis when treated with STS, we investigated whether this was associated with the failure to activate caspase-2, -3, and -7. The experiments presented in Fig. 5, A-C show that no proteolytic cleavage of these caspases was detectable in cell lysates obtained after 1, 3, 6, 12, and 24 h of treatment with 4 mM STS. Similar results were also obtained after treatment with NPA (data not shown).

**Cleavage of PARP and DFF.** An increasing number of downstream targets of caspase-3 and -7 have been identified. We focused our attention on PARP, a repair enzyme that is cleaved and inactivated by caspase-3 and -7 (18), and DFF (19) or ICAD (20, 21), the cleavage of which is necessary for the activation of the caspase-activated DNase (CAD; Ref. 20) that is responsible for internucleosomal DNA degradation during apoptosis. Treatment with NPA and STS was associated with cleavage of the substrates PARP and DFF/ICAD (Fig. 6). In LNCaP-Bcl-2 cells, in which STS (Fig. 1C) and NPA (data not shown) did not induce apoptosis, no cleavage of PARP or DFF/ICAD (Fig. 5, D and E) was observed.

**Inhibition of Caspase-3 and -7 Activation.** To further link caspase-3 and -7 activation to apoptosis of LNCaP cells, cells were treated for 12 h with STS ± the caspase inhibitors z-VD-FMK or z-DEVd-FMK. Although treatment with the panspecific z-VD-FMK completely inhibited STS-induced DNA laddering (Fig. 7A) and TUNEL positivity (STS-treated cells: 45 ± 3% TUNEL-positive cells; STS- and z-VD-treated cells: 8 ± 1.6% TUNEL-positive cells; vehicle-treated cells: 4.1 ± 0.3% TUNEL-positive cells), treatment with the more narrowly specific z-DEVd-FMK minimally inhibited DNA laddering (Fig. 7A) and TUNEL (STS- and z-DEVd-treated cells: 37 ± 4% TUNEL-positive cells; STS-treated cells: 45 ± 3% TUNEL-positive cells). The different ability of the two inhibitors to prevent apoptosis was probably due to their different effectiveness in preventing caspase-3 and -7 activation. This is shown in the Western analysis of caspase-3, -7, DFF, and PARP obtained from lysates of LNCaP cells treated for 12 h with STS ± z-VD-FMK or z-DEVd-FMK (Fig. 7, B–E). Although z-DEVd-FMK completely prevented the processing of caspase-3 and -7, as well as the cleavage of the two substrates DFF and PARP, z-DEVd-FMK was only partially effective in this regard (Fig. 7, B–E). These experiments showed conclusively that prevention of apoptosis occurred only when caspase-3 and -7 activation was completely inhibited.

**Mechanisms of Apoptosis Inhibition.** Because the accumulation of cytochrome c in the cytosol is a fundamental event mediating the progression of apoptosis in other systems (15, 16), we investigated whether the inhibition of STS-induced apoptosis with z-VD-FMK or z-DEVd-FMK could be related to cytochrome c release.
Bcl-2 overexpression was associated with alterations in the intracellular distribution of cytochrome c, and in the prevention of \( \Delta \psi_m \) changes. Fig. 2C shows that z-VAD-FMK, similarly to Bcl-2 overexpression (Fig. 2E), prevented changes of the transmembrane potential of LNCaP cells treated with STS for 4 h. However, z-VAD-FMK was unable to prevent STS-induced cytochrome c translocation into the cytosol, whereas this phenomenon was successfully inhibited by Bcl-2 overexpression (Fig. 8, A and B, respectively). These experiments demonstrated that Bcl-2 overexpression or treatment with z-VAD-FMK prevented STS-induced apoptosis with different mechanisms of action. Bcl-2 overexpression blocked cytochrome c translocation and subsequent caspase activation, whereas z-VAD-FMK worked further downstream by direct inhibition of caspase activation.

Apoptosis Induced by Viral-mediated Overexpression of Caspase-7 in LNCaP and LNCaP-Bcl-2 Cells. Since caspase-7 is activated in every model of apoptosis that we have characterized thus far, we wished to learn whether overexpression of this protease could directly cause apoptosis of LNCaP cells. These experiments were done by infecting LNCaP cells with Av-C7 or Av-lacZ. The resulting transiently infected cells were named LNCaP-C7 and LNCaP-lacZ. Cells were harvested at 24, 48, 72, and 96 h and analyzed for caspase-7 expression and processing, DEVDase activity, and TUNEL. Induction of caspase-7 protein overexpression and processing was present only in cells infected with Ad-C7 (Fig. 9B), and was evident at 48 h postinfection. There was a 7.5-fold induction of DEVDase activity that became evident after 48 h and remained constant throughout the experiment in cells infected with Ad-C7 (Fig. 9A). The percentage of apoptotic cells was 15.5 ± 0.7% and 80 ± 3% after 72 and 96 h postinfection, respectively, in LNCaP-C7. There was a 1.8 ± 0.2% and a 6.5 ± 1% background apoptotic index in LNCaP-lacZ cells at 72 and 96 h, respectively (Fig. 9C).

Similar experiments were performed with the cell line LNCaP-Bcl-2, and the resulting cell lines were named LNCaP-Bcl-2-C7 and LNCaP-Bcl-2-lacZ. Again, infection with Av-C7 was specifically associated with the induction of caspase-7 protein expression and processing (Fig. 10B), DEVDase activity (5.5-fold above LNCaP-Bcl-2-lacZ; Fig. 10A), and TUNEL (Fig. 10C). Overexpression of caspase-7 peaked at 72 h postinfection and was 45-fold higher than in LNCaP-Bcl-2-lacZ (Fig. 10B). The apoptotic index in LNCaP-Bcl-2-C7 cells was 38 ± 2% at 96 h and was 3-fold higher than LNCaP-Bcl-2-lacZ cells (Fig. 10C).

DISCUSSION

Proteolytic Activation of Caspase-3 and -7 Is a Common Event Leading to Apoptosis of LNCaP Cells. Two substances with different mechanisms of action—NPA (a phosphomevalonate inhibitor) and STS (an inhibitor of several protein kinases and topoisomerase II)—induced the apoptosis of LNCaP cells by similar signaling pathways.
Shortly after treatment with these agents, we observed the release of cytochrome c from the mitochondria to the cytosol, activation of caspase-3 and -7, and cleavage of the downstream targets of activated caspases, PARP and DFF/ICAD. When the pan-caspase inhibitor z-VAD-FMK was used to prevent caspase-3 and -7 activation, STS and NPA were unable to induce apoptosis of LNCaP cells and cleavage of the downstream targets PARP and DFF/ICAD. A similar scenario was observed in LNCaP-Bcl-2 cells, in which resistance to STS and NPA-induced apoptosis was associated with the lack of caspase-3 and -7 activation or PARP and DFF/ICAD cleavage. These observations, together with a previous report in which we observed that lovastatin induces apoptosis of LNCaP cells by activating caspase-7 (7), have delineated the central role played by the caspases (in particular caspase-7) in mediating apoptosis of LNCaP cells. The mechanism through which STS and NPA activate the apoptotic pathway is still undetermined. Interestingly, a lower dose of STS inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced apoptosis of LNCaP cells by interfering with the protein kinase C pathway (22). The reason why we (and many others) have observed induction of apoptosis by STS is probably related to additional effects that STS may have when used at 80-fold higher concentration [i.e., inhibition of other protein kinases (23) and/or topoisomerase II (24) activity].

z-VAD-FMK and Bcl-2 Overexpression Prevent Apoptosis with Different Mechanisms of Action. Translocation of cytochrome c into the cytosol is one of the first events occurring in many cells undergoing apoptosis (9, 25). This phenomenon is followed by the proteolytic activation of the early caspases in the apoptosome (26), a complex of apoptogenic factors consisting of the mammalian CED-4 homologue APAF-1 (27), caspase-9 (28, 29), cytochrome c, and dATP (30). It has been suggested that during apoptosis, cytochrome c accumulates into the cytosol in which it binds Apaf-1 and induces conformational changes that allow Apaf-1 to bind caspase-9, which in turn is cleaved into its active subunits (30, 31). dATP is thought to provide the energy necessary for the activation of caspase-9 (30). Caspase-9, or other cell type-specific upstream caspases, is the earliest caspase activated in nonreceptor-mediated apoptosis (30). Active caspase-9 then cleaves and activates other downstream caspases, including caspase-3 and -7, which sets in motion the execution phase of apoptosis (30).

In LNCaP cells, apoptosis induced by STS or NPA was averted by z-VAD-FMK treatment or Bcl-2 overexpression. Although both methods of inhibition prevented activation of caspase-3 and -7, the mechanisms were different. Bcl-2 prevented translocation of cytochrome c to the cytosol and, consequently, the activation of the early caspase(s) that at this time are unidentified for LNCaP cells. z-VAD-FMK directly prevented the proteolytic activation of the caspases (32) but did not prevent the translocation of cytochrome c to the cytosol. Notably, both Bcl-2 overexpression and treatment with z-VAD-FMK prevented changes in the Δψm. These observations agree with some (9) but not other reports (33) and imply that cytochrome c transloca-
tion does not require a mitochondrial transmembrane depolarization and that the decrease of the ΔΨm is a consequence of caspase activation. An important conclusion derived from these experiments is that the prevention of caspase activation is a mechanism-independent event necessary for the prevention of apoptosis in LNCaP cells and presumably in other prostate cancer cells.

Bcl-2 Is a Global Inhibitor of Apoptosis That Is Overexpressed in Prostate Cancer. Prostate cancer arises from the secretory cells of prostatic epithelium, and Bcl-2 is a well characterized antiapoptotic factor that may play an important role in this disease. In benign prostatic epithelium, Bcl-2 is expressed in the basal cell layer but not in the secretory cells (34–36). The pattern of Bcl-2 expression in prostate cancer varies with disease progression. Before androgen ablation, Bcl-2 is not expressed or is heterogeneously expressed (36, 37) in many patients with prostate cancer. However, in a subset of such patients, Bcl-2 is present and predicts poor survival (38–40). In primary or metastatic specimens obtained from individuals failing androgen ablation, Bcl-2 is frequently overexpressed (36, 37, 41). Thus, these observations identify Bcl-2 expression as a possible prognostic risk for patients with prostate cancer. Two mechanisms could explain the accumulation of cells expressing this oncoprotein. Bcl-2 expression could be stimulated by androgen ablation so that the

Fig. 6. Cleavage of PARP and DFF in LNCaP cells undergoing apoptosis after treatment with STS or NPA. Immunoblot analysis of PARP (A and C) and DFF (B and D) in LNCaP cell lysates obtained during treatment with STS [4 μM, 0–24 h (A and B)] or NPA [10 μM, 0–72 h (C and D)]. The processing of PARP is indicated by the progressive disappearance of the Mₐ 115,000 (115 kDa) full-length protein and by the progressive appearance of the Mₙ 85,000 (85 kDa) cleaved fragment. Cleavage of DFF is indicated by the progressive disappearance of the Mₐ 45,000 (45 kDa) and Mₙ 40,000 (40 kDa) subunits. The migration of the fragments of PARP and DFF differs from that in Fig. 4 because two different gel apparatuses were used in the two experiments (Figs. 4 and 6), which accounts for a longer migration in the experiment shown in Fig. 6.

Fig. 7. Prevention of STS-induced apoptosis of LNCaP cells is elicited by the caspase inhibitor z-VAD-FMK but not z-DEVD-FMK. A, agarose gel electrophoresis of genomic DNA extracted from LNCaP cells 12 h after treatment with vehicle alone (Control), STS (4 μM) + z-VAD-FMK (100 μM), STS (4 μM) + z-DEVD-FMK (100 μM), or STS (4 μM). MW, molecular weight markers. B–E, immunoblot analysis of caspase-3, -7, DFF, and PARP, respectively, in LNCaP cell lysates after 12 h of treatment with vehicle alone (Control), STS + z-DEVD-FMK (4 and 100 μM), STS + z-VAD-FMK (4 and 100 μM), and STS alone (4 μM). Processing of caspase-3, DFF, and PARP is indicated by the disappearance of the major band. Processing of caspase-7 is indicated by the disappearance of the major band and by the appearance of the Mₐ 20,000 (20kDa) subunit. In C, there is a Mₐ 20,000 (20kDa) band also in the cells treated with vehicle alone, which indicates some baseline activation of caspase-7. When STS was given alone, the intensity of the Mₐ 20,000 (20kDa) band increased significantly, which indicates an accumulation of the active form of caspase-7 after treatment with STS.

Fig. 8. Cytochrome c translocation to the cytosol is inhibited by Bcl-2 overexpression but not by treatment with z-VAD-FMK. A, immunoblot analysis of cytochrome c in cytosolic lysates from LNCaP cells extracted after 12 h of treatment with vehicle alone, STS (4 μM), or STS + z-VAD-FMK (4 and 100 μM, respectively). B, cytochrome c expression in cytosolic lysates from LNCaP-Bcl-2 cells after treatment with vehicle alone or STS (4 μM for 12 and 24 h).

Fig. 8. Cytochrome c translocation to the cytosol is inhibited by Bcl-2 overexpression but not by treatment with z-VAD-FMK. A, agarose gel electrophoresis of genomic DNA extracted from LNCaP cells 12 h after treatment with vehicle alone (Control), STS (4 μM) + z-VAD-FMK (100 μM), STS (4 μM) + z-DEVD-FMK (100 μM), or STS (4 μM). MW, molecular weight markers. B–E, immunoblot analysis of caspase-3, -7, DFF, and PARP, respectively, in LNCaP cell lysates after 12 h of treatment with vehicle alone (Control), STS + z-DEVD-FMK (4 and 100 μM), STS + z-VAD-FMK (4 and 100 μM), and STS alone (4 μM). Processing of caspase-3, DFF, and PARP is indicated by the disappearance of the major band. Processing of caspase-7 is indicated by the disappearance of the major band and by the appearance of the Mₐ 20,000 (20kDa) subunit. In C, there is a Mₐ 20,000 (20kDa) band also in the cells treated with vehicle alone, which indicates some baseline activation of caspase-7. When STS was given alone, the intensity of the Mₐ 20,000 (20kDa) band increased significantly, which indicates an accumulation of the active form of caspase-7 after treatment with STS.
tissue can resist androgen ablation-induced apoptosis. Such a possibility is suggested by the observation that castration up-regulates the expression of prostatic Bcl-2 mRNA in mice (37) and of Bcl-2 immunoreactivity in prostatic tissue obtained before and after androgen ablation in patients with prostate cancer (42) or benign prostatic hyperplasia (35). Alternatively, it could be that cells already overexpressing Bcl-2 are selected by androgen ablation, in part, for their ability to resist apoptosis and, in part, for their increased malignant phenotype. Such possibilities are suggested by the observation that Bcl-2 overexpression in LNCaP cells confers an androgen-independent (8) and a metastatic (43) phenotype. Down-regulation of Bcl-2 expression may have therapeutic benefits in some circumstances. For instance, antisense oligos for this oncoprotein have been described to decrease its expression and reconstitute sensitivity to etoposide-induced apoptosis in LNCaP cells (44).

Our studies with LNCaP-Bcl-2 cells have been helpful in understanding the molecular bases of cell-death resistance in prostate cancers overexpressing this oncoprotein and have shown that Bcl-2 overexpression prevents the activation of caspase-3 and -7 while also preventing the accumulation of cytochrome c in the cytosol. Because caspase activation occurs downstream from the Bcl-2 check point (45), this observation has clinical implications and suggests that activation of the caspases can induce apoptosis in prostate cancers that overexpress Bcl-2.

**Overexpression of Caspase-7 Induces Apoptosis of LNCaP and LNCaP-Bcl-2 Cells.** The observation that STS-induced apoptosis of LNCaP cells occurs if caspase-3 and -7 are activated implies that these caspases are potential therapeutic targets to repress or to induce apoptosis. Manipulation of the apoptotic pathway for the treatment of human diseases is becoming an important field of investigation inasmuch as many conditions associated with excess or impaired PCD have been described (46).

Prevention or enhancement of caspase activity for therapeutic purposes has already been demonstrated in many animal disease models. The damage observed in the central nervous system of animals undergoing cerebral ischemia can be minimized by using inhibitors of caspase activation in an acute setting (47–49). Similar results have been obtained in animal models of myocardial ischemia (50, 51). Yu et al. (52) induced therapeutic apoptosis of a rat glioma model using a retrovirus containing the cDNA of caspase-1. Kondo et al. (53) successfully induced apoptosis of human and murine malignant glioma cells in vivo and in vitro after retroviral transfer of caspase-3 and -6. Additional evidence supports the potential tumoricidal ability of the caspases. On transfection and subsequent overexpression, many of these proteases can induce apoptosis of the host cell (28, 54–56). Other caspases, including caspase-7, require the removal of the NH2-terminal prodomain to effectively induce apoptosis of the host cell (10, 57, 58).

Because caspase-7 was an active participant in every model of apoptosis of LNCaP cells that we have developed thus far and the prevention of its activation by z-VAD-FMK or Bcl-2 was associated with the prevention of apoptosis, we hypothesized that LNCaP and LNCaP-Bcl-2 cells would undergo apoptosis after retroviral transfer of caspase-3 and -6. Additional evidence supports the potential tumoricidal ability of the caspases. On transfection and subsequent overexpression, many of these proteases can induce apoptosis of the host cell (28, 54–56). Other caspases, including caspase-7, require the removal of the NH2-terminal prodomain to effectively induce apoptosis of the host cell (10, 57, 58).

Because caspase-7 was an active participant in every model of apoptosis of LNCaP cells that we have developed thus far and the prevention of its activation by z-VAD-FMK or Bcl-2 was associated with the prevention of apoptosis, we hypothesized that LNCaP and LNCaP-Bcl-2 cells would undergo apoptosis after the overexpression of this protease. A caspase-7 cDNA was incorporated into a first generation adenoviral vector in which the expression of the gene of interest is under the control of the powerful RSV promoter. Overexpression of caspase-7 protein paralleled by a concomitant increase of DEVD-ase activity was seen after 48 h, and apoptosis was evident by 72 h postinfection. Importantly, overexpression of caspase-7 was able to bypass the antiapoptotic activity of the oncoprotein Bcl-2 in the cell line LNCaP-Bcl-2.

These experiments have several implications. They have demonstrated that the naturally occurring caspase pathway can be manipulated in an in vitro setting to induce apoptosis of a prostate cancer cell line generated from a patient affected by androgen-independent prostate cancer. More importantly, it seems that caspase overexpression...
has the ability to bypass the antiapoptotic effect of the oncoprotein Bcl-2, a putative mediator of apoptosis resistance in androgen-independent prostate cancer.

The observations described in this paper provide the theoretical foundation for a new and promising therapeutic approach for prostate cancer. Adenovirus-mediated overexpression of caspase-7 may be a useful gene therapy approach to treat advanced prostate cancer. It remains to be seen whether caspase overexpression is a feasible approach to induce apoptosis in vivo, where the growth of prostatic epithelium is under the physiological stimulation of endocrine and paracrine growth factors and where the anatomical boundaries with the adjacent structures (i.e., the prostatic stroma) are intact. Future studies will address these issues as well as the need to limit caspase (over)expression to prostatic epithelium by using tissue-specific promoters.

ACKNOWLEDGMENTS

We thank Drs. Buttyan (Columbia University, New York, NY), Wang (University of Texas Southwestern Medical Center, Dallas, TX), and Dixit (Genentech, San Francisco, CA) for reagents. NPA was a gift from Dr. W. Perkins, Elan Pharmaceutical Research Corporation (Gainesville, GA).

REFERENCES


Signaling Pathway Activated during Apoptosis of the Prostate Cancer Cell Line LNCaP: Overexpression of Caspase-7 as a New Gene Therapy Strategy for Prostate Cancer

Marco Marcelli, Glenn R. Cunningham, Margaret Walkup, et al.