Caspase-7 Is Activated during Lovastatin-induced Apoptosis of the Prostate Cancer Cell Line LNCaP

Marco Marcelli, Glenn R. Cunningham, S. Joe Haidacher, Sebastian J. Padayatty, Lydia Sturgis, Carolina Kagan, and Larry Denner

Departments of Medicine [M. M., G. R. C., S. J. H., S. J. P.] and Cell Biology [M. M., G. R. C.], Veterans Affairs Medical Center and Baylor College of Medicine, Department of Cell Biology [L. S., C. K., L. D.], Texas Biotechnology Corporation, Houston, Texas 77030

ABSTRACT

The goals of this work were to establish a reproducible and effective model of apoptosis in a cell line derived from advanced prostate cancer and to study the role of the caspase family of proteases in mediating apoptosis in this system. The study involved the use of the prostate cancer cell line LNCaP. Apoptosis was induced using the hydroxymethyl glutaryl CoA reductase inhibitor, lovastatin, and was evaluated by agarose gel electrophoresis of genomic DNA, morphological criteria, and terminal deoxynucleotidyl transferase-mediated nick end labeling. Caspases were studied by catalytic activity, mRNA induction, and protein processing. Lovastatin (30 μM) was an effective inducer of apoptosis, causing changes that were evident after 48 h and essentially complete after 96–120 h of treatment. These effects were prevented by the simultaneous addition of mevalonate (300 μM) to the culture medium. Lovastatin induced a proteolytic activity that was able to cleave the enzyme poly(ADP-ribose) polymerase and the substrate Z-DEVD-AFC, which is modeled after the tetrapeptide P1-P4 amino acids of the poly(ADP-ribose) polymerase cleavage site. Caspase-7, but not caspase-3, underwent proteolytic activation during lovastatin-induced apoptosis, an effect prevented by mevalonate. Caspase-7 was the only detected interleukin 1β converting enzyme family protease with DEVD cleavage activity that exhibited lovastatin-induced mRNA up-regulation. Again, mevalonate blocked this effect. Lovastatin-induced apoptosis also was prevented when the caspase inhibitors Z-DEVD-CH2F or Z-VAD-CH2F (100 μM) where added to the medium.

These studies have identified lovastatin as a powerful inducer of apoptosis in the cell line LNCaP. Caspase activation was a necessary event for LNCaP cells to undergo apoptosis during treatment with lovastatin. Of the caspases tested, only caspase-7 underwent proteolytic activation after stimulation with lovastatin. Identification of caspase-7 as a potential mediator of lovastatin-induced apoptosis broadens our knowledge of the molecular events associated with programmed cell death in a cell line derived from prostatic epithelium.

INTRODUCTION

Prostate cancer remains the second leading cause of cancer deaths in American men and the number of patients predicted to die from prostate cancer in 1997 accounts for 14% of all cancer deaths in males (1). Hormonal or androgen-ablative therapy (i.e., orchietomy, diethylstilbestrol, luteinizing-hormone releasing-hormone analogues, and androgen receptor antagonists) is the predominant systemic treatment for metastatic prostate cancer (2). Although an initial response to hormonal therapy is observed in 70–80% of patients with advanced disease, most tumors progress rapidly to androgen-independent growth, and only 10–20% of the patients are alive 5 years following initiation of treatment (3). Prostate cancer is composed of clones of androgen-dependent and androgen-independent cells even before therapy (4). The initial response following androgen ablation is thought to be due to the induction of apoptosis of androgen-dependent prostate cancer cells (5). Because androgen-independent cells are insensitive to androgen ablation, they remain alive even in the absence of androgens (6). Hence, after the initial response to androgen ablation due to the death of androgen-dependent cells, the tumor progresses to an androgen-independent state, and the patient is no longer curable (7). Although androgen-independent prostate cancers are resistant to androgen ablation-induced apoptosis, these cells still retain the basic machinery required for programmed cell death (8). To implement effective treatment for advanced prostate cancer, it is, therefore, very important to understand the molecular events responsible for apoptosis of prostatic epithelium.

The LNCaP cell line is one of the most widely investigated prostate cancer cell lines. This cell line originated from a lymph node metastasis of a patient who had undergone androgen ablation by castration after failing treatment with oral estrogens (9). LNCaP cells do not undergo apoptosis when cultured in steroid-deficient medium (10), and they contain a well-characterized mutation of the androgen receptor (11).

We have used LNCaP cells to develop an in vitro model of apoptosis. Our goal was to understand the molecular events associated with programmed cell death in a prostate cancer cell line. Apoptosis was induced in LNCaP cells with the cholesterol-lowering agent lovastatin (12). Candidate molecules responsible for apoptosis in LNCaP cells were searched among the caspases, a family of proteases that are important effectors of apoptosis (13). We demonstrated a connection between lovastatin-induced apoptosis of LNCaP cells and the proteolytic activation of the interleukin 1β converting enzyme-like protease caspase-7 (14).

MATERIALS AND METHODS

Materials. Lovastatin was a gift of Dr. Y-S. Chao (Merck Research Laboratories, Rahway, NJ). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). S1 nuclease was obtained from Boehringer Mannheim (Indianapolis, IN). Chemicals, protease inhibitors, and mevalonate were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum and tissue culture media were purchased from Life Technologies, Inc. (Gaithersburg, MD).

The caspase inhibitors Ac-YVAD-CHO and Ac-DEVD-CHO were from Biomol (Plymouth Meeting, PA). The caspase inhibitors, Z-VD-CH2F and Z-DEVD-CH2F, and the fluorogenic substrates, Z-YVAD-AFC and Z-DEVD-AFC, were from Enzyme System (Dublin, CA). [α-32P]UTP was from ICN Radiochemicals (Irvine, CA). The enhanced chemiluminescence detection reagents were from Amersham Corp. (Arlington Heights, IL).

Cell Culture. LNCaP cells (obtained from American Type Culture Collection) were grown in RPMI 1640 containing 10% fetal bovine and 1% penicillin-streptomycin. Different concentrations of lovastatin were added to the medium for varying lengths of time (specified in the text). The inactive lactone of lovastatin was converted to the active form after solubilization in ethanol and the addition of an equimolar amount of NaOH. After an incubation at 60°C for 90 min, ethanol was evaporated under nitrogen, and the sodium salt was solubilized in ethanol and stored at −20°C. Fresh lovastatin, dissolved at

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a concentration 1000-fold higher than the working concentration, was added every 24 h. Controls received ethanol vehicle at a concentration equal to that in lovastatin-treated cells.

**Apoptosis Assays.** During lovastatin treatment, cells detached from the base of the tissue culture plate. Therefore, cells adherent to the dish and floating in the tissue culture medium were both present at some time points in these experiments. Adherent cells were harvested by trypsinization. Floating cells were harvested by sedimentation at 3200 × g for 1 min. For DNA fragmentation analyses, adherent and floating cells were combined. Genomic DNA was extracted using the Easy DNA kit (Invitrogen, San Diego, CA), and sized in a 2% agarose gel. Apoptosis was indicated by the appearance of the typical DNA fragmentation pattern, or laddering (15).

Apoptosis also was determined using TUNEL-3 (16). In these experiments, adherent and floating cells were harvested separately, transferred to glass slides using a Cyto-Tek centrifuge (Miles, Elkart IN), air dried, and fixed with 4% formaldehyde in PBS for 30 min. After washing with PBS, cells were incubated with the TUNEL reaction mixture (Boehringer) at 37°C for 1 h. Slides were washed six times with PBS, mounted in Mowiol, viewed under fluorescence and phase microscopy, and photographed at ×50 using Kodak Gold 400 film. Apoptosis was indicated by the appearance of brightly labeled nuclei and apoptotic bodies. Apoptosis also was determined morphologically (17) using phase contrast photomicroscopy before and after 48 h of stimulation with 30 μM lovastatin.

**Western Analysis.** For the immunodetection of PARP (18), adherent and floating cells were harvested after 0, 24, 36, 48, 54, 60, 72, and 96 h of treatment with 30 μM lovastatin, resuspended in SDS-PAGE loading buffer A [62.5 mM Tris (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 0.003% bromphenol blue, and 5% β-mercaptoethanol], and sonicated for 20 s. An aliquot of the floating cells were treated for 30 μM lovastatin, resuspended in SDS-PAGE loading buffer A [62.5 mM Tris (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 0.003% bromphenol blue, and 5% β-mercaptoethanol] at 24—120 h. The apoptotic pattern appeared after 72 h. At 96 and 120 h, the apoptotic process was more evident; however, a substantial amount of high molecular weight DNA was still unprocessed. In C, cells were treated with 30 μM lovastatin for 24—120 h. The apoptotic pattern appeared by 48 h, and the process was almost complete at 72 h, when no residual high molecular weight DNA was visible. D, cells were treated for 0—120 h with 30 μM lovastatin and 300 μM mevalonate. Mevalonate prevented the apoptotic effects of lovastatin.
RESULTS

Agarose Gel Electrophoresis. To identify the dose of lovastatin that induces apoptosis, LNCaP cells were cultured for 5 days with 0.3 nm to 30 μM lovastatin. Gel electrophoresis analysis demonstrated cleavage of genomic DNA only at 3 and 30 μM lovastatin (Fig. 1A). At 3 μM lovastatin, DNA laddering was minimal after 72 h and evident at 96 and 120 h. However, a substantial amount of intact high molecular weight DNA was still present (Fig. 1B). The kinetics of lovastatin-induced apoptosis were different at 30 μM. At this concentration, DNA laddering became evident after 48 h, and the process of DNA fragmentation was basically complete after 72 h, when there was almost no intact high molecular weight DNA left (Fig. 1C). Because 30 μM lovastatin induced apoptosis more efficiently than 3 μM, the higher dose was used for most of the subsequent experiments.

Fig. 4. Lovastatin induces cleavage of the “death” substrate PARP. Cell lysates were collected from LNCaP cells after treatment with 30 μM lovastatin for 0, 24, 36, 48, 54, 60, 72, and 96 h (Lanes 1–8, respectively). PARP cleavage (indicated by the appearance of a M, 85,000 fragment) was evident after 48 h (Lane 4).

As, 5′-GCACCACTGGGTTAGGTTTCTAG-3′. Total RNA extracted from LNCaP cells was annealed with random hexamers and reverse transcribed with murine leukemia virus reverse transcriptase as suggested by the manufacturer (GeneAmp RNA PCR kit; Perkin-Elmer, Branchburg, NJ). The resulting cDNA was then amplified using the relevant pair of primers by PCR (35 cycles of annealing-extension at 68°C for 60 s and of denaturation at 95°C for 60 s). The amplified product was subcloned in the pCR4-TOPO plasmid (Invitrogen, San Diego CA) and sequenced by dideoxy sequencing. In each instance, there was 100% homology to the published sequences of these caspases.

S5 Protection Assay. Total RNA was extracted from combined adherent and floating LNCaP cells after treatment with lovastatin. Ten μg of total RNA were used in S5 protection assays performed as reported previously using [α-32P]UTP-labeled cRNA probes (26). Quantitative analysis was performed by densitometry using the software program NIH Image 1.59. The intensity of the protected bands for caspase-1, -3, -6, -7, and -9 (of 275, 264, 321, 333, and 330 nucleotides in size, respectively) was corrected for the intensity of the protected band corresponding to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase analyzed in the same sample (26).

Inhibition of Caspase Activity. Inhibition of caspases with DEVD cleavage activity is achievable in vivo using the inhibitors Z-DEVD-CH2F and Z-VDAD-CH2F (21, 27). In these experiments, 10,000 cells were seeded in a 12-well plate on day 0. Z-DEVD-CH2F or Z-VDAD-CH2F were added to the medium at the concentration of 100 μM 24 h before the addition of lovastatin (30 μM). Control wells were treated with lovastatin, Z-DEVD-CH2F, or Z-VDAD-CH2F alone. After 48 h, cells were harvested and processed as described previously for TUNEL.

Fig. 2. TUNEL of adherent and floating LNCaP cells treated with 30 μM lovastatin for 0–96 h. The percentage of cells undergoing apoptosis and of cells floating in the tissue culture medium increased over time. By 96 h, essentially all cells were floating in the medium and undergoing apoptosis.

Fig. 3. Activation of proteases with DEVD cleavage activity during lovastatin-induced apoptosis. A minor peak of DEVD cleavage activity was present in the lysate of adherent cells between 48 and 72 h of treatment with lovastatin. Much greater activity was present in floating cells between 48 and 96 h. YVAD cleavage activity in the adherent cells was low, did not increase during 96 h of treatment, and was undetectable in floating cells.

Assessment of Catalytic Activity. Because little is known of the role played by the caspases in prostatic tissue undergoing apoptosis, we have investigated whether lovastatin-induced apoptosis of LNCaP
A small peak of DEVD cleavage activity was detectable in lysates of adherent cells between 48 and 72 h of treatment with lovastatin. Considerably more DEVD cleavage activity was detected in the floating cells. This was evident at 48 h of treatment and persisted for the entire length of the experiment. Interestingly, DEVD cleavage activity paralleled the appearance and progression of apoptosis detected by agarose gel electrophoresis (Fig. 1C) and TUNEL (Fig. 2). These data indicated that lovastatin-induced apoptosis of LNCaP cells was associated with the activation of DEVD cleavage activity and that most of this activity was localized in the floating cells.

PARP Cleavage. A common feature of the cysteine proteases with DEVD cleavage activity is the ability to cleave the enzyme PARP into \( M_f 85,000 \) and \( M_i 25,000 \) fragments (18). To study whether PARP cleavage activity was present in LNCaP cells treated with lovastatin, lysates derived from adherent and floating cells that had been treated with 30 \( \mu \)M lovastatin for 0–96 h were analyzed with a well-characterized monoclonal antibody that recognizes the native \( M_f 116,000 \) PARP protein as well as the \( M_i 85,000 \) fragment (18). As shown in Fig. 4, an \( M_f 85,000 \) cleavage product appeared after 48 h of treatment with lovastatin. The appearance of the cleavage product paralleled both the appearance and progression of apoptosis detected by agarose gel electrophoresis (compare Fig. 4 and Fig. 1C). PARP cleavage was inhibited when LNCaP cells were treated simultaneously with lovastatin and mevalonate (data not shown).

Analysis of Cysteine Proteases with the Ability to Cleave the Substrate DEVD and PARP. Cysteine proteases with the ability to cleave the substrate DEVD and the enzyme PARP include caspase-3 (28), -6 (24), -7 (14), and -9 (25). Caspase-1 also can cleave PARP when added at stoichiometric concentrations (a feature shared with caspase-2 and -4; Refs. 20, 29, and 30); however, when these pro-
teases are added at catalytic concentrations, they probably are not effective. We sought to determine whether any of these caspases mediates lovastatin-induced apoptosis by analyzing mRNA and protein expression in LNCaP cells treated with lovastatin.

**RNA Analysis.** mRNAs for caspase-1, -3, -6, -7, and -9 were analyzed by S1 protection assay of total RNA extracted from LNCaP cells treated in the absence and presence of 30 μM lovastatin for 48 h, the time for initial appearance of apoptosis seen by agarose gel electrophoresis in Fig. 1C. Basal mRNA levels for caspase-1 (Fig. 5A), caspase-3 (Fig. 5B), caspase-6 (Fig. 5C), and caspase-9 (Fig. 5D) were very low (Lane 1), and no stimulation with lovastatin was observed (Lane 2). Caspase-7 mRNA was increased 2.5-fold after 48 h of treatment with 30 μM lovastatin (Fig. 5E, Lane 2), an effect prevented by simultaneous treatment with mevalonate (Fig. 5E, Lane 3). To determine whether induction of caspase-7 mRNA was important for the induction of apoptosis in our model, we performed an S1 protection assay of total RNA obtained from LNCaP cells treated with concentrations of lovastatin ranging from 3 nM to 30 μM (Fig. 5F, Lanes 1–6). Because apoptosis was detected at 48 h of treatment only in cells that received 30 μM lovastatin (Fig. 1, B and C), we hypothesized that if induction of caspase-7 mRNA was relevant to lovastatin-induced apoptosis, it should be induced only in cells receiving 30 μM lovastatin. Fig. 5F, Lane 6, shows that caspase-7 mRNA was induced only by 30 μM lovastatin.

**Western Analysis.** During apoptosis, the caspases are activated by cleavage into two subunits (21). Western analysis was performed on lysates of LNCaP cells treated with 30 μM lovastatin for 0 and 96 h to learn whether caspase-3 or -7 were cleaved during the course of lovastatin-induced apoptosis. Caspase-7 was cleaved and processed into its active form after 4 days of treatment with lovastatin (Fig. 6A). This was indicated by the appearance of a Mr 30,000 band, representing an intermediate cleavage product of caspase-7 in which the prodomain has been removed from the full-length molecule, and by a band of Mr 20,000, representing the active subunit p20 that contains the antibody epitope. Caspase-3 remained in its inactive form for the entire period of treatment (Fig. 6C), and no changes in the intensity of its signal were evidenced by the treatment. Caspase-7 cleavage was prevented by the addition of mevalonate (Fig. 6B), adding further support for the hypothesis that caspase-7 was a mediator of lovastatin-induced apoptosis.

**Inhibition of Caspase Activity.** To determine whether caspase activation is central to lovastatin-induced apoptosis, lovastatin was administered to LNCaP cells that had been treated with Z-DEVD-CH2F or Z-VAD-CH2F, two powerful inhibitors of caspase activity. Apoptosis was completely prevented when either inhibitor (100 μM) was added to the media 24 h prior to the addition of lovastatin (30 μM; Fig. 7, compare B with C and D). Control LNCaP cells were unaffected by the addition of either inhibitor alone (data not shown).

**DISCUSSION**

Our studies have demonstrated that lovastatin induces apoptosis of the prostate cancer cell line LNCaP, and that the interleukin 1β converting enzyme-like protease caspase-7 is one of the mediators of this effect. The critical importance of the caspases in mediating lovastatin-induced apoptosis was demonstrated by the observation that programmed cell death was prevented not only by mevalonate, which bypasses the metabolic block imposed by lovastatin, but also by the simultaneous administration of two powerful inhibitors of caspase activity, Z-VAD-CH2F and Z-DEVD-CH2F.

The ability of lovastatin to induce apoptosis has been documented in other cell lines (31–33). Other cell lines treated in our laboratory with this cholesterol-lowering agent undergo apoptosis, including other prostate cancer cell lines, stromal cells cultured from the prostate of a patient with benign prostatic hyperplasia (34), and Jurkat and HL60 cells (data not shown). We used this cholesterol-lowering agent to induce apoptosis in LNCaP cells because in our hands it was much more effective than other known inducers of apoptosis, such as cycloheximide, etoposide, or serum starvation.

The mechanism(s) whereby lovastatin triggers apoptosis is unclear. Because lovastatin inhibits hydroxymethyl glutaryl CoA reductase, metabolites of the cholesterol pathway, such as farnesyl and geranyl PP, that are needed for isoprenylation of critical proteins (31), are reduced. Lamin A, an important constituent of the nuclear membrane, is one protein that may be impacted by lovastatin because it requires farnesylation to reach its final mature form (35). Lamin A is recognized as a substrate that is degraded during apoptosis (36), and its cleavage may be a prerequisite for apoptotic nuclear disassembly (27). Ras is another protein that may trigger the apoptotic response in cells...
treated with lovastatin, because it is prevented from attaining its mature conformation after treatment with this hydroxymethyl glutaryl CoA reductase inhibitor (31).

Lovastatin blocks cell growth at the early G1-phase of the cell cycle (37). This is associated with an elevation of the cyclin-dependent kinase inhibitors p27 and p21 (38, 39). We have found an induction of p21 mRNA and protein also in LNCaP cells (data not shown). Because this induction was present also at concentrations of lovastatin (0.3 nM to 300 nM) that do not induce apoptosis (data not shown), we think that p21 induction mediates G1 arrest of the cell cycle but not apoptosis.4

The caspases are important mediators of the final steps of apoptosis. At least 10 members of this family have been isolated (40). Their genes encode proteins that are translated as inactive proenzymes. These precursors are activated by cleavage after specific aspartate residues and assembly into heterotetramers. The mature heterotetramer proteolyses specific substrates after aspartate residues. The proteolytic targets of activated caspases can be classified into three groups. The first group includes the inactive precursors of various family members. The second group includes homeostatic or structural enzymes such as PARP (18), lamin A (36), the sterol regulatory element binding proteins (41, 42), α-fodrin, and others (reviewed in Ref. 43). The third group includes molecules that are activated into their mature form upon cleavage by the caspases. Two identified members of this group are DNA fragmentation factor (44), which mediates DNA cleavage, and p21-activated kinase 2, which mediates some of the morphological changes that are seen in apoptotic cells (45). Hence, once the caspases became active, they exert their apoptotic activity in part by autoactivation, in part by cleaving substrates that are critical for cell survival and in part by activating downstream effectors.

The data in this report provide the first evidence for involvement of a caspase in apoptosis of a prostate cancer cell line. Although the exact sequence of events during activation of the different cysteine proteases is not known, caspase-7 is widely recognized to be one of the downstream effectors of this pathway (46). On the basis of sequence homology, caspase-7 is classified as a member of the subgroup of cysteine proteases most related to the Caenorhabditis elegans factor CED-3, which also includes caspase-3, -6, and -9 (25). Caspase-7 can be processed into its mature form by caspase-3 (14), caspase-8 (47), or caspase-10 (46). Caspase-8 and -10 are two cysteine proteases located upstream in the pathway of caspase activation and contain NH2-terminal regions of high homology with the death domain of the Fas-associated death domain protein (46, 48). Because caspase-3 is not cleaved during lovastatin-induced apoptosis, it is

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4 M. Marcelli, unpublished observations.
likely that caspase-8, -10, or an unknown protease is responsible for the proteolytic activation of caspase-7 in our system.

The major challenge for the treatment of prostate cancer is to find agents that can specifically eliminate androgen-independent prostate cancer cells. Lovastatin is a powerful inducer of apoptosis in cultured cell lines; however, in vivo use of lovastatin for this purpose is unlikely. In clinical practice, a 40-mg dose of lovastatin produces a peak blood level of 55 ng/ml (100 nM), a concentration 200-fold lower than that used in this study to induce apoptosis (49). Because many cell lines undergo apoptosis when treated with 30 µM lovastatin, it is likely that systemic use at such high doses would be associated with intolerable side effects.

A complete understanding of the molecular mechanisms regulating the apoptotic pathway in prostate cancer is relevant for developing more effective treatments of this disease. Androgen ablation initially produces a dramatic reduction in the tumor burden in most patients, presumably because androgen-dependent cells undergo apoptosis. However, expression of the antiapoptotic protein, Bcl-2, is increased in hormone-refractory prostate cancer (50, 51). Therefore, it is conceivable that the initial shrinkage of the tumor after androgen ablation may be due to the induction of apoptosis in androgen-dependent cells, and that failure to eliminate the entire tumor burden may be due to the presence of a mechanism that resists androgen ablation-induced apoptosis in androgen-independent cells. Our experimental model may allow identification of caspases that can overcome such resistance. Overexpression of such caspases using gene therapy may provide a means for activating endogenous pathways to eliminate androgen-independent prostate cancer in humans. Although these experiments have shown that the activation of the caspases is central to lovastatin-induced apoptosis and that caspase-7 is proteolytically activated during this phenomenon, additional studies will clarify whether this event is common to other prostate cancer cell lines and to other models of apoptosis of prostatic epithelium.

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