Raloxifene, a Selective Estrogen Receptor Modulator, Induces Apoptosis in Androgen-responsive Human Prostate Cancer Cell Line LNCaP through an Androgen-independent Pathway

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Abstract

Raloxifene, a selective estrogen receptor (ER) modulator, is a mixed estrogen agonist/antagonist that has been shown to prevent osteoporosis and breast cancer in women. Because the prostate contains a high level of ER-β, the present study investigated the effect of raloxifene in the androgen-sensitive human prostate cancer cell line LNCaP. Previously, it has been demonstrated that LNCaP cells express ER-β but not ER-α and that tamoxifen induces apoptosis in these cells. After treatment with raloxifene, a dramatic increase in cell death occurred in a dose-dependent manner (10⁻⁹ to 10⁻⁶ M range). Using the terminal deoxynucleotidyl transferase-mediated nick end labeling apoptotic assay, we demonstrated that the nuclear fragmentation was due to apoptosis. The dramatic change in cellular morphology after treatment with raloxifene was no longer observed when cells were pretreated with a pan-caspase inhibitor, Z-VAD-FMK, and a specific caspase-9 inhibitor, Z-LEHD-FMK. Furthermore, immunoblot demonstrated an activation of caspase-9 in LNCaP cells. Because LNCaP cells contain a mutated androgen receptor that allows cellular proliferation in the presence of antiandrogens, prostate-specific antigen assay and transfection with a reporter construct containing luciferase gene under the control of androgen response element (pARE) were carried out. The results demonstrated that raloxifene does not significantly alter androgen receptor activity in LNCaP cells. Taken together, these results demonstrate that raloxifene, a selective ER modulator, induces apoptosis in the androgen-sensitive human prostate cancer cell line LNCaP through an androgen-independent pathway.

Introduction

In the United States, prostate cancer is the most common malignancy and the second leading cause of male cancer deaths (1). Despite the success of early screening with PSA, approximately 30% of prostate cancer patients who undergo radical prostatectomy or radiotherapy for clinically localized disease go on to develop either a local recurrence and/or a distant relapse (2, 3). Because chemotherapy remains ineffective in curing metastatic prostate cancer, surgical or medical castration continues to be the therapy of choice for treating recurrence following the primary therapy for prostate cancer. After androgen withdrawal, PSA levels decrease dramatically, and bone pain is generally alleviated. However, prostate cancer cells inevitably become resistant to the androgen withdrawal therapy, commonly within a median time of 18–24 months (4). Once hormone-refractory prostate cancer emerges, treatment is largely limited to palliative care.

Raloxifene is the prototypical SERM that has been shown to prevent osteoporosis and breast cancer (5, 6). Currently, the agent has Food and Drug Administration approval for the treatment and prevention of osteoporosis. Although raloxifene binds to both ER-α and -β with high affinity (7, 8), the binding affinity to ER-α is four times higher than that to ER-β. Among the well-known SERMs, which include tamoxifen, drolxifene, and idoxifene, raloxifene is unique in that it is an estrogen antagonist in the uterus (9). In the breast and bone, all SERMs act as estrogen antagonists and agonists, respectively (10). The mechanism for the observed tissue-specific effect of SERMs is currently unknown.

Since the initial cloning of ER-β from the rat prostate cDNA library by Küpper et al. (11), there has been a line of evidence that suggests that estrogen and its receptor are important in the prostate. First, it was demonstrated by immunohistochemistry in the rat prostate that ER-α is present in the stroma, whereas ER-β is preferentially localized in the epithelium (12). Second, an increased expression of ER-α has been associated with prostate cancer progression and metastasis and eventual emergence of hormone-refractory phenotype (13). Third, a recent Phase II clinical trial using the estrogen agonist diethylstilbestrol as a second-line hormonal therapy demonstrated a >50% decrease in the level of PSA in 43% of the patients (14). Fourth, ER-β knockout mice exhibit prostatic hyperplasia (15). Lastly, tamoxifen has been shown to induce programmed cell death in LNCaP cells (16). These observations, taken together, suggest that ER is a potential target for therapeutic intervention in prostate cancer patients. Therefore, the present study examined the effect of raloxifene in the androgen-responsive human prostate cancer cell line LNCaP. We report that raloxifene induces apoptosis in these cells through an androgen-independent pathway.

Materials and Methods

Cell Culture and Mitogenic Assay. LNCaP was purchased from American Type Culture Collection (Manassas, VA). All cells used in this study were from the 35th through 40th passages. Cells were routinely maintained in RPMI 1640 containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). Raloxifene and tamoxifen were diluted to 10⁻⁶ M in 70% ethanol and added to the culture medium at the indicated concentrations. The androgen analogue, R1886, was purchased from NEN Life Science Products Inc. (Boston, MA) and diluted to 10⁻⁸ M. Subsequently, cells were treated with 10⁻⁸ M R1886 for the indicated amount of time.

For cell counts, cells were plated at 20,000 cells/well in 24-well culture plates in RPMI 1640 supplemented with 10% FBS and allowed to adhere for 24 h. Then the cultures were washed two times with PBS, and the cells from previously selected wells were counted to determine the plating efficiency.
Cells in the remaining wells were cultured for 4 days in phenol-red-free RPMI 1640 supplemented with 1% charcoal-stripped FBS containing raloxifene at 0, 10⁻¹¹, 10⁻⁹, 10⁻⁷, 10⁻⁵, and 10⁻³ m. Raloxifene was added so that the ratio of 70% ethanol to the culture medium was 1:1000. For control, 70% ethanol was added to culture at 1:1000. The medium was changed at day 2. After removing the medium and detaching the cells with 0.5 ml of 0.05% trypsin, cells were counted using a hemocytometer. Photomicrographs were taken to document the changes in cellular morphology. All experiments were repeated three times, and similar results were obtained each time.

Cell proliferation assay using tamoxifen was carried out in a manner identical to that used for raloxifene, with the exception of the dosages. Tamoxifen was used at 0, 10⁻⁷, 10⁻⁵, 10⁻³, and 10⁻¹ m.

**Flow Cytometry Analysis.** For flow cytometry assay, LNCaP cells were grown in 6-well plates and incubated for 24 h at 37°C in the presence or absence of 10⁻⁶ m raloxifene. Cells were harvested and then washed twice with PBS buffer (pH 7.4). After fixing in 80% ethanol for 30 min, cells were washed twice and resuspended in PBS (pH 7.4) containing 0.1% Triton X-100, 5 μg/ml PI, and 50 μg/ml RNase A for DNA staining. Cells were then analyzed by a FACScan cytometer (program CELLQUEST; Becton Dickinson); red fluorescence due to PI staining of DNA was expressed on a logarithmetic scale simultaneously to the forward scatter of the particles. Four thousand events were counted on the scatter gate. The number of apoptotic nuclei was expressed as a percentage of the total number of events.

**Immunoblot Analysis.** Cells were harvested and lysed using lysis buffer (0.0625 n Trizma base, 2% SDS, and 5% 2-mercaptoethanol). After the concentration of protein was determined using the Bradford assay, the samples were boiled for 10 min, and electrophoresis was carried out using 50 μg of total protein in each lane. After electrophoresis, protein was transferred to a 0.2-μm nitrocellulose membrane. After the transfer, nonspecific binding sites were blocked by incubation in TBST for 1 h. Subsequently, membranes were incubated with TBST containing the appropriate antibody at a dilution of 1:1000 overnight at 4°C. Antibodies against caspase-9 (Asp353) and ER-α and ER-β were purchased from Cell Signaling Technology Inc. (Beverly, MA) and Upstate Biotechnology (Lake Placid, NY), respectively. After washing with TBST, membranes were incubated in the presence of rabbit antimouse secondary antibody (Pierce Chemical Co., Rockford, IL) at a dilution of 1:3000 for 2 h, and immunoreactive bands were visualized by enhanced chemiluminescence.

**TUNEL Assay and Caspase Inhibitor Treatment.** Cells were plated on chamber slides and incubated 24 h before treatment with raloxifene. After exposure to raloxifene for a designated amount of time, cells were fixed in 4% paraformaldehyde (pH 7.4) for 10 min. TUNEL assay of fragmented DNA was performed as recommended by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN).

Z-VAD-FMK (Roche Molecular Biochemicals), DEVD-CHO (Calbiochem-Novabiochem Corp., San Diego, CA), and Z-LEHD-FMK (Calbiochem-Novabiochem Corp.) were dissolved in DMSO to a concentration of 50 mM and then added to medium at 50 μM 30 min before treatment with raloxifene. As a control, DMSO was added to the culture medium at 1:1000. Cells were observed for 24 h, and photomicrographs were taken intermittently to document the changes in cellular morphology.

**Transient Transfection and Luciferase Activity Assay.** Cells were seeded in 6-well plates at 100,000 cells/well. After cells were allowed to adhere, they were transiently transfected with 1 μg of pARE, the plasmid containing the luciferase reporter gene under the control of ARE (17) using 12 μl of Lipofectin in 1 ml of transfection medium (Opti-mem; Life Technologies, Inc.) according to the manufacturer’s directions. After 24 h of incubation, fresh medium was added, and cells were incubated for an additional 24 h. Finally, varying combinations of R1886 (Sigma, St. Louis, MO) and raloxifene were added, and the cultures were maintained for an additional 4 h. The extent of the promoter activity of pARE was assayed by measuring luciferase activity using a commercial luciferase assay kit (Enhanced Luciferase Assay Kit; Analytical Luminescence Laboratory, San Diego, CA). Luciferase activity was normalized with protein concentration determined by the Bradford assay.

**PSA Assay.** Cells were seeded in 24-well plates at 30,000 cells/well. After cells were allowed to adhere, medium was changed to phenol-red-free RPMI 1640 supplemented with 1% charcoal-stripped FBS. Twenty-four h later, varying combinations of R1886 and raloxifene were added to the wells, and cells were cultured for 4 h. Subsequently, conditioned medium was collected and centrifuged briefly to remove any cellular debris. The level of secreted PSA was determined using the ultrasensitive chemiluminescence assay on a DPC 2000 Immunoassay Analyzer (Diagnostic Product Co., Los Angeles, CA). PSA levels were normalized against the number of cells present in the wells after the 4-h culture period.

**Statistics.** All numerical data are expressed as mean ± SE with either triplicate or quadruplicate observations. Differences between the means of different treatments were compared by χ². P < 0.05 was considered statistically significant.

**Results.**

**Effect of Raloxifene in LNCaP Cells.** Initially, reverse transcription-PCR was carried out to determine the status of ER expression in LNCaP cells. Lau et al. (18) reported previously that LNCaP cells expressed ER-β but not ER-α. Our results also demonstrated that LNCaP cells express only ER-β (data not shown). To confirm the results of reverse transcription-PCR, Western blot analysis was carried out for ER-β. As expected, ER-β was detected in LNCaP cells (Fig. 1).

To determine the effect of raloxifene, cell numbers were determined after treatment with increasing doses of raloxifene for 4 days. For comparison, LNCaP cells were also treated with tamoxifen (Fig. 2C). The results, shown in Fig. 2B demonstrated that raloxifene inhibited the proliferation of LNCaP cells in a dose-dependent manner. The minimum concentration of raloxifene needed to detect a significant decrease in cell number was 10⁻⁶ m. The cell count at 10⁻³ m raloxifene was approximately 15% of control. With the decrease in cell count, a dramatic change in cellular morphology that was suggestive of cell death was also observed. As shown in Fig. 2A, the normal morphology of LNCaP cells, a typical spindle shape with occasional pseudopodium-like extensions, was observed in the presence of ethanol only. However, alteration in the morphology of these cells appeared when they were treated with raloxifene. The observed changes included shrinkage of the cytoplasm and increased frequency of cytosolic vacuole. After the treatment with 10⁻³ m raloxifene for 3–6 h, numerous intracellular vesicles followed by floating cells appeared in the culture. For comparison, human breast cancer cells were also treated with raloxifene. It was observed that the profile of sensitivity to raloxifene is similar between the prostate and the breast cancer cells, demonstrating that the effect of raloxifene is likely ER-specific in human prostate cancer cells (data not shown).

**Raloxifene-induced Cell Death in Human Prostate Cancer Cell Lines.** To further characterize the nature of cell death induced by raloxifene in LNCaP cells, measurement of nuclear incorporation of PI by FACS analysis and TUNEL apoptotic assay was carried out. As shown in Fig. 3A, raloxifene treatment induced a population of apo-

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**Fig. 1.** Western blot analysis for ER-β in LNCaP cells. PC3M cells were used as a positive control.

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Please note that the text is already in plain text format and does not require further conversion. The document is a scientific research paper discussing the effects of raloxifene on LNCaP cells, including cell proliferation, morphology changes, and apoptosis induction through various methodologies. The results indicate that raloxifene inhibits cell proliferation and induces apoptosis in LNCaP cells, with dose-dependent effects and similar sensitivity profiles as observed in human breast cancer cells.
totic nuclei. Likewise, the TUNEL apoptotic assay showed a dramatic increase in the number of dark brown positive cells, demonstrating a significant increase in the rate of apoptosis in a time-dependent manner after raloxifene treatment in LNCaP cells (Fig. 3, B and C).

Because caspase activation is usually necessary for apoptosis, cells were treated with the pan-caspase inhibitor Z-VAD-FMK before raloxifene treatment. The results revealed that the dramatic change in cellular morphology induced by raloxifene was no longer observed after the addition of Z-VAD-FMK to the culture medium (Fig. 4A). To define whether a particular caspase(s) plays the critical role in raloxifene-induced apoptosis, specific inhibitors were used. The caspase-3 inhibitor DEVD-CHO (Ref. 19; 10 μM) was able to partially block cell death induced by raloxifene, whereas the caspase-9-specific inhibitor Z-LEHD-FMK (Ref. 20; 20 μM) almost completely blocked raloxifene-induced apoptosis (Fig. 4B). To further demonstrate that caspase-9 is involved in raloxifene-induced apoptosis, immunoblot analysis was carried out for caspase-9. Raloxifene treatment caused activation of caspase-9 in LNCaP cells (Fig. 4C). These results suggest that the raloxifene-induced apoptosis in LNCaP cells is mediated through caspases.

Raloxifene and Androgen. Because androgen receptor in LNCaP cells contains a mutation in the ligand binding domain (21), the potential interaction between raloxifene and androgen receptor was investigated. To this end, cells were transiently transfected with pARE, a construct containing ARE in association with the luciferase reporter gene. Subsequently, cells were treated with varying doses of raloxifene in the presence and absence of R1886, an androgen analogue. Because raloxifene caused a significant change in cellular morphology in <6 h, cells were exposed to raloxifene for only 4 h. The results, shown in Fig. 5A, demonstrated that raloxifene does not significantly change the level of luciferase activity. To further investigate the relationship between androgen receptor and raloxifene, we measured the PSA level in conditioned media after treatment with increasing doses of raloxifene. When PSA levels were normalized for the number of cells, we again observed no significant difference (Fig. 5B). These results, taken together, demonstrate that raloxifene does not significantly alter androgen receptor activity or function in LNCaP cells.

Discussion

The results of the present study demonstrated that the SERM raloxifene induces cell death in the androgen-responsive human pros-
raloxifene-induced apoptosis in prostate cancer cell line LNCaP. FACS analysis, TUNEL assay, caspase inhibitor treatment, and immunoblot for caspase-9 demonstrated that the observed cell death after raloxifene treatment is apoptosis-mediated by caspase cascade. Finally, PSA assay and transient transfection experiments using an androgen-responsive luciferase reporter construct demonstrated that raloxifene acts independent of androgen signaling. These results, taken together, provide a valuable insight concerning the role of estrogen/ER in prostate cancer cells and suggest raloxifene as a potential treatment in prostate cancer patients.

Although the precise role of estrogen and its receptors in benign and malignant prostatic epithelial cells has not been established, there is a body of evidence that suggests that estrogens and its receptors are important regulators in the prostate. First, the level of expression of ER-β is high in the prostate (22). In fact, ER-β was originally cloned from a rat prostate cDNA library (11). Second, ER-β knockout mice have prostate and bladder hyperplasia (15). Third, Bonkhoff et al. (13) demonstrated an association between increased levels of expression of ER-α and the hormone-refractory/metastatic phenotype of prostate cancer after examining samples obtained from six patients with hormone-refractory cancer and two patients with metastatic disease. In the present study, we were able to reproduce the results of an earlier report by Lau et al. (18), who reported that androgen-responsive LNCaP cells express ER-β but not ER-α. The same report also demonstrated that most androgen-independent human prostate cancer cell lines express varying combinations of ER-α and ER-β (18). These consistent expressions of ERs in human prostate cancer cell lines suggest that estrogen/ERs may be potential targets for therapeutic intervention in prostate cancer patients.

Raloxifene, a SERM that binds to both ER-α and ER-β with high affinity (7, 8), is a mixed estrogen agonist/antagonist. Clinical investigations have demonstrated that it is a safe agent for prevention of both osteoporosis and breast cancer (5, 6). In the present study, it was demonstrated that raloxifene causes cell death in a dose- and time-dependent manner in androgen-sensitive LNCaP cells. This observed cell death was shown to be apoptosis using multiple approaches. Since the initial description of apoptosis, it has been demonstrated that there are multiple pathways for programmed cell death. Currently, three basic apoptotic signaling pathways have been established: (a) mitochondria; (b) endoplasmic reticulum; and (c) death receptor (23). The three pathways are similar in that they eventually involve activation of caspases; however, the subtype of caspases activated appears to differ significantly among the varying apoptotic pathways. In the present study, the activation of caspase-9 has been demonstrated in LNCaP cells. The activation of caspase was demonstrated to be critical for raloxifene-induced apoptosis because treatment with the pan-caspase inhibitor Z-VAD-FMK and the specific caspase-9 inhibitor Z-LEHD-FMK before exposure to raloxifene prevented cell death.

The raloxifene-induced apoptosis in LNCaP cells is likely ER mediated because LNCaP has been shown to respond to estrogen in vitro (18). Alternatively, the presence of high-affinity binding sites for SERMs that are independent of ER binding has been demonstrated in multiple systems (24, 25). Because these ER-independent SERM binding sites are usually more abundant than ER, it is possible that the observed raloxifene-induced apoptosis is mediated in an ER-independent manner in human prostate cancer cells. Further work is necessary to verify this hypothesis.

The possibility exists that raloxifene-induced apoptosis may be mediated through androgen receptor in LNCaP cells. It has been shown previously that LNCaP has mutated androgen receptor that permits cellular proliferation in the presence of antiandrogens such as flutamide (21). In the present study, however, it was demonstrated that raloxifene does not significantly alter the activity of androgen-responsive promoter or PSA secretion, demonstrating that raloxifene does not interfere with androgen signaling. These results also suggest that targeting the ER with raloxifene along
with androgen ablation may increase the rate of prostate cancer cell death and ultimately improve outcome in patients with prostate cancer. A clinical trial is warranted to verify this concept.

In conclusion, the present study demonstrated that raloxifene, a SERM, induces apoptosis in the androgen-responsive human prostate cancer cell line LNCaP. Because raloxifene has been shown to be effective with an acceptable side effect profile in preventing both breast cancer and osteoporosis in women (5, 6), the present results suggest that raloxifene is a viable agent for clinical trials in treating and preventing prostate cancer. The use of raloxifene for both the treatment and prevention of prostate cancer will now be investigated.

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References

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