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Raloxifene, a Mixed Estrogen Agonist/Antagonist, Induces Apoptosis in Androgen-independent Human Prostate Cancer Cell Lines

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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 high levels of ER-β, the present study investigated the effect of raloxifene in three well-characterized, androgen-independent human prostate cancer cell lines: (*a*) PC3; (*b*) PC3M; and (*c*) DU145. Reverse transcriptase-PCR and Western blot analysis for ER-α and ER-β demonstrated that all three cell lines express ER-β, whereas only PC3 and PC3M cells were positive for ER-α. After the treatment with raloxifene, a dramatic increase in cell death was observed in a dose-dependent manner in the three prostate cancer cell lines (10^{-9} to 10^{-6} M range). Because the three prostate cancer cell lines demonstrated similar morphological changes after the raloxifene treatment, PC3 (ER-α/ER-β+) and DU145 (ER-β+ only) cells were selected to further characterize the raloxifene-induced cell death. Using the nucleus-specific stain 4′,6-diamidino-2-phenylindole, nuclear fragmentation was observed in a time-dependent manner in both cell lines after exposure to 10^{-6} M raloxifene. Using the terminal deoxynucleotidyl transferase-mediated nick end labeling apoptotic assay, it was demonstrated that the nuclear fragmentation was caused by apoptosis. To investigate the possibility that caspase activation is involved in raloxifene-induced apoptosis, cells were treated with the pan-caspase inhibitor ZVAD. The results demonstrated that the dramatic change in cellular morphology after treatment with raloxifene was no longer observed when cells were pretreated with ZVAD. Immunoblot demonstrated activation of caspases 8 and 9 in PC3 and DU145 cells, respectively. Taken together, these results demonstrate that the mixed estrogen agonist/antagonist, raloxifene, induces apoptosis in androgen-independent human prostate cancer cell lines.

INTRODUCTION

In the United States, prostate cancer is the most common malignancy and the second leading cause of male cancer deaths (1). Since the widespread implementation of PSA[2] for prostate cancer screening in the late 1980s, the incidence of nonorgan-confined prostate cancer has decreased dramatically (2). Nevertheless, ~30% of prostate cancer patients who undergo radical prostatectomy or radiotherapy for clinically localized disease go on to develop either a local or distant relapse (3, 4). Once prostate cancer recurs after the definitive treatment for a localized disease, the most widely used treatment is either a medical or a surgical androgen withdrawal; chemotherapy has been shown to be largely ineffective in treating prostate cancer. In addition to patients with recurrence, androgen ablation is also the treatment of choice for those who present with extensive local invasion or distant metastasis. After androgen withdrawal, there is a dramatic decrease in PSA with improvement in clinical parameters. However, prostate cancer cells inevitably become resistant to the androgen-withdrawal therapy with a median time of 18–24 months (5). Once the 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 (6, 7); other well-known members of SERMs include tamoxifene, droloxifene, and idoxifene. SERMs are synthetic estrogen ligands that can exhibit either an estrogenic or an antiestrogenic effect depending on the tissue types (reviewed in Refs. 8 and 9). Specifically, SERMs are usually ER agonists in bone, liver, and cardiovascular system; ER antagonists in brain and breast; and mixed ER agonists/antagonists in the uterus (reviewed in Refs. 8 and 9). Published works have demonstrated that raloxifene binds to both ER-α and ER-β with high affinity (10, 11); however, the binding affinity to ER-α is four times higher than ER-β. Among the SERMs, raloxifene is unique in that it is an estrogen antagonist in the uterus (12). In the breast and bone, although, all SERMs act as estrogen antagonists and agonists, respectively (13). The mechanism for the observed tissue-specific effect of SERMs has been shown recently to be attributable to differences in coregulator recruitment in a tissue-specific manner (14).

Since the discovery of ER-β from the rat prostate cDNA library (15), series of evidence have suggested an important role for estrogen/ER in the prostate: (*a*) in the rat prostate, immunohistochemistry has demonstrated that ER-α is present in the stroma, whereas ER-β is localized preferentially in the epithelium (16); (*b*) increased expression of ER-α has been associated with prostate cancer progression, metastasis, and hormone-refractory phenotype (17); (*c*) a recent Phase II clinical trial using the estrogen agonist diethylstilbestrol in hormone-refractory prostate cancer demonstrated >50% decrease in the level of PSA in 43% of the patients (18); and (*d*) ER-β knockout mice exhibit prostate and bladder hyperplasia (19). These observations, taken together, suggest that ER is a reasonable target for therapeutic intervention in prostate cancer patients. Therefore, the present study examined the effect of raloxifene in androgen-independent human prostate cancer cell lines. We report that raloxifene induced apoptosis in androgen-independent human prostate cancer cells.

MATERIALS AND METHODS

Cell Culture and Mitogenic Assay. Human prostate (PC3 and DU145) and breast (MCF-7, ZR-75–1, and HS-578T) cancer cell lines were purchased from American Type Culture Collection (Rockville, MD). PC3M cells were kindly provided by Dr. Jane Trepel (National Cancer Institute, NIH). All cells used in this study were from 35th through 40th passages. Cells were routinely maintained in RPMI 1640 containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). Raloxifene (Eli-Lily, Indianapolis, IN) was diluted to 10^{-2} M in 70% ethanol and added to the culture medium at selected concentrations.

For cell counts, cells were plated at 20,000/well in 24-well culture plates in RPMI 1640 supplemented with 10% FBS and allowed to adhere for 24 h. Then

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[2] The abbreviations used are: PSA, prostate-specific antigen; ER, estrogen receptor; RT-PCR, reverse transcription-PCR; DAPI, 4′,6-diamidino-2-phenylindole; FBS, fetal bovine serum; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; TBS, Tris-buffered saline; SERM, selective estrogen receptor modulator.

the cultures were washed two times with PBS, and the cells from wells selected previously 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^{-6}, and 10^{-5} M. Raloxifene was added such 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 hemocytometer. Photomicrographs were taken to document the changes in cellular morphology. All experiments were repeated three times, and similar results were obtained each time.

RNA Isolation and RT-PCR. RT-PCR for ER-α and ER-β was carried out as described previously (17). Cells were harvested, and total RNA was isolated using TRIzol reagent (Life Technologies, Inc., Grand Island, NY), according to the manufacturer's protocol. Once isolated, total RNA was reverse transcribed using Superscript (Life Technologies, Inc.) and random hexamer using the following conditions: 42°C for 50 min and 70°C for 15 min. After reverse transcription, the samples were incubated with RNase H for 30 min at 37°C. Subsequently, PCR amplification was performed as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 35 cycles followed by 10-min incubation at 72°C. The following primers were used: ER-α, 5′ primer–tactgcatcagatccaaggg, 3′ primer–gtgggaatgatgaaaggtgg; ER-β, 5′ primer–tgaaaaggaaggttagtgggaacc, 3′ primer–tggtcagggacatcatcatgg; glyceraldehyde-3-phosphate dehydrogenase, 5′ primer–accacagtccatgccatcac, 3′ primer–tccaccaccctgttgctgta. To visualize the PCR products, the samples were subjected to electrophoresis in 1% agarose gel followed by staining with ethidium bromide. The authenticity of the products was confirmed by Southern blot analysis.

Nuclear Staining. Cells were plated on glass slides and treated with raloxifene for varying lengths of time. Subsequently, they were fixed in cold 3.5% paraformaldehyde for 5 min and washed with PBS once. After permeabilizing for 2 min with methanol at −20°C, cells were rinsed with PBS and incubated for 5 min in 50 m[scap]m glycine in PBS. After another wash with PBS, the slides were mounted with the mounting media Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Nuclear morphology was visualized using con-focal microscopy.

Immunoblot Analysis. Cells were harvested and lysed using lysis buffer (0.0625 N Trizma base, 2% SDS, and 5% 2-mercaptoethanol). After determining the concentration of protein using 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 (Invitrogen, La Jolla, CA). After the transfer, the membranes were incubated in blocking buffer TBST (5% nonfat dry milk, TBS, and 0.1% Tween) for 1 h. Subsequently, the membranes were incubated with TBST-containing appropriate antibodies at a dilution of 1:1000 overnight at 4°C. Antibodies against caspases 8 and 9 and ER-α and –β were purchased from Upstate Biotechnology (Lake Placid, NY). After washing with TBST, the 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. After washing several times with TBST, immunoreactive bands were visualized by enhanced chemiluminescence.

TUNEL and ZVAD 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).

ZVAD (Roche Molecular Biochemicals) was dissolved in DMSO to a concentration of 50 mM. Then it was added to medium at 50 μM 30 min before treatment with raloxifene. As control, DMSO was added to the culture medium at 1:1000. Cells were observed for 48 h, and photomicrographs were taken to document the changes in cellular morphology.

Statistics. All numerical data are expressed as mean ± SE with triplicate observations. Differences of the means among different treatments were compared by χ^2. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Raloxifene in Androgen-independent Human Prostate Cancer Cell Lines. RT-PCR was initially carried out to determine the status of ER expression in the three androgen-independent human prostate cancer cell lines: (*a*) PC3; (*b*) PC3M; and (*c*) DU145. Authenticity of the PCR products was confirmed by Southern blot analysis. The results demonstrate that only PC3 and PC3M cells expressed ER-α, whereas all three cell lines were positive for ER-β (data not shown). To confirm the results of RT-PCR, immunoblot for ER-α and -β was subsequently carried out (Fig. 1*a*). As expected, all three cell lines expressed ER-β, whereas only PC3 and PC3M had detectable levels of ER-α. These results are consistent with previous reports which demonstrated that PC3 cells express both ER-α and -β, whereas DU145 cells had only ER-β (20).

To determine the effect of raloxifene in the four human androgen-independent prostate cancer cell lines, cell numbers were determined after treatment with increasing doses of raloxifene for 4 days. The result, shown in Fig 1*b*, demonstrated that raloxifene inhibited the proliferation of all three prostate cancer cell lines in a dose-dependent manner. The minimum concentration of raloxifene needed to detect a significant decrease in cell number was 10^{-9} M. The cell count at 10^{-6} M raloxifene was ~5, 25, and 20% of that of control in DU145, PC3, and PC3M cells, respectively. At 10^{-5} M raloxifene, the cell counts of all three cell lines were <10%

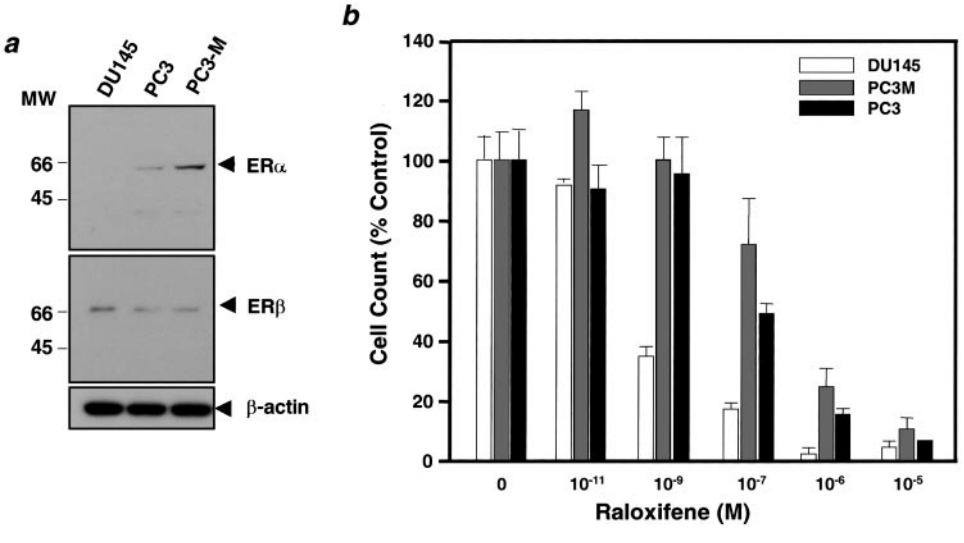

Fig. 1. Effect of raloxifene on androgen-independent human prostate cancer cell lines. *a*, immunoblot analysis for ERs in human prostate cancer cell lines. As expected, all three prostate cancer cell lines were positive for ER-β, whereas only PC3 and PC3M expressed ER-α. *b*, cell count. There were significant decreases in cell numbers in a dose-dependent manner.

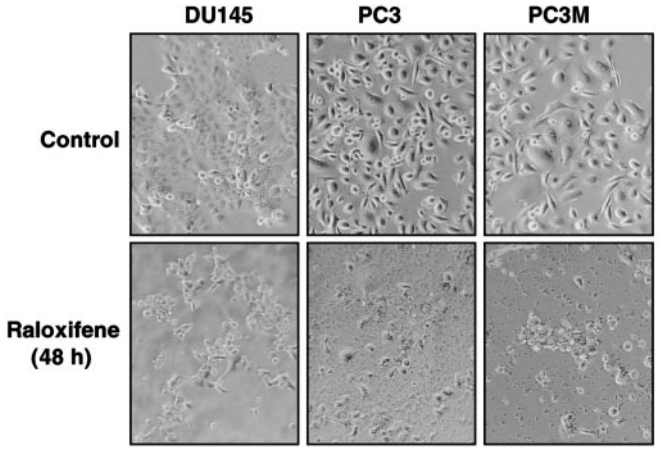

Fig. 2. Effect of raloxifene on cellular morphology. In association with the significant decrease in cell count, a dramatic change in cellular morphology suggestive of cell death appeared after treatment with raloxifene.

of control. Interestingly, the ER-α-positive cells, PC3 and PC3M, were more resistant to raloxifene than DU145 cells.

With the decrease in cell count, dramatic changes in cellular morphology suggestive of cell death were also observed (Fig. 2). The observed changes included shrinkage of cytoplasm and increased frequency of cytosolic vacuoles. After the treatment with 10^{-6} M raloxifene for 48 h, numerous intracellular vesicles followed by floating cellular debris appeared in the culture. For comparison, MCF-7 and HS-578T human breast cancer cells were also treated with raloxifene. RT-PCR demonstrated that MCF-7 cells express ER-α, whereas HS-578T cells have undetectable levels of expression of both ER-α and -β. As expected based on the ER expression profile, raloxifene significantly decreased the rate of proliferation in MCF-7 cells but not that of HS-578T cells. It was observed that the profile of sensitivity to raloxifene is similar between the prostate and the MCF-7 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 investigate the possibility that the raloxifene-induced cell death in human prostate cancer cell lines is apoptosis, PC3 (ER-α and -β positive) and DU145 (ER-β positive) were selected for further analysis. The following experiments were all carried out at 10^{-6} M raloxifene, because this concentration induced near complete cell death in all three cell lines. Initially, nuclear staining using DAPI was carried out to determine the nuclear morphology after treatment with raloxifene at 10^{-6} M. As shown in Fig. 3*a*, a time-dependent increase in nuclear fragmentation was observed.

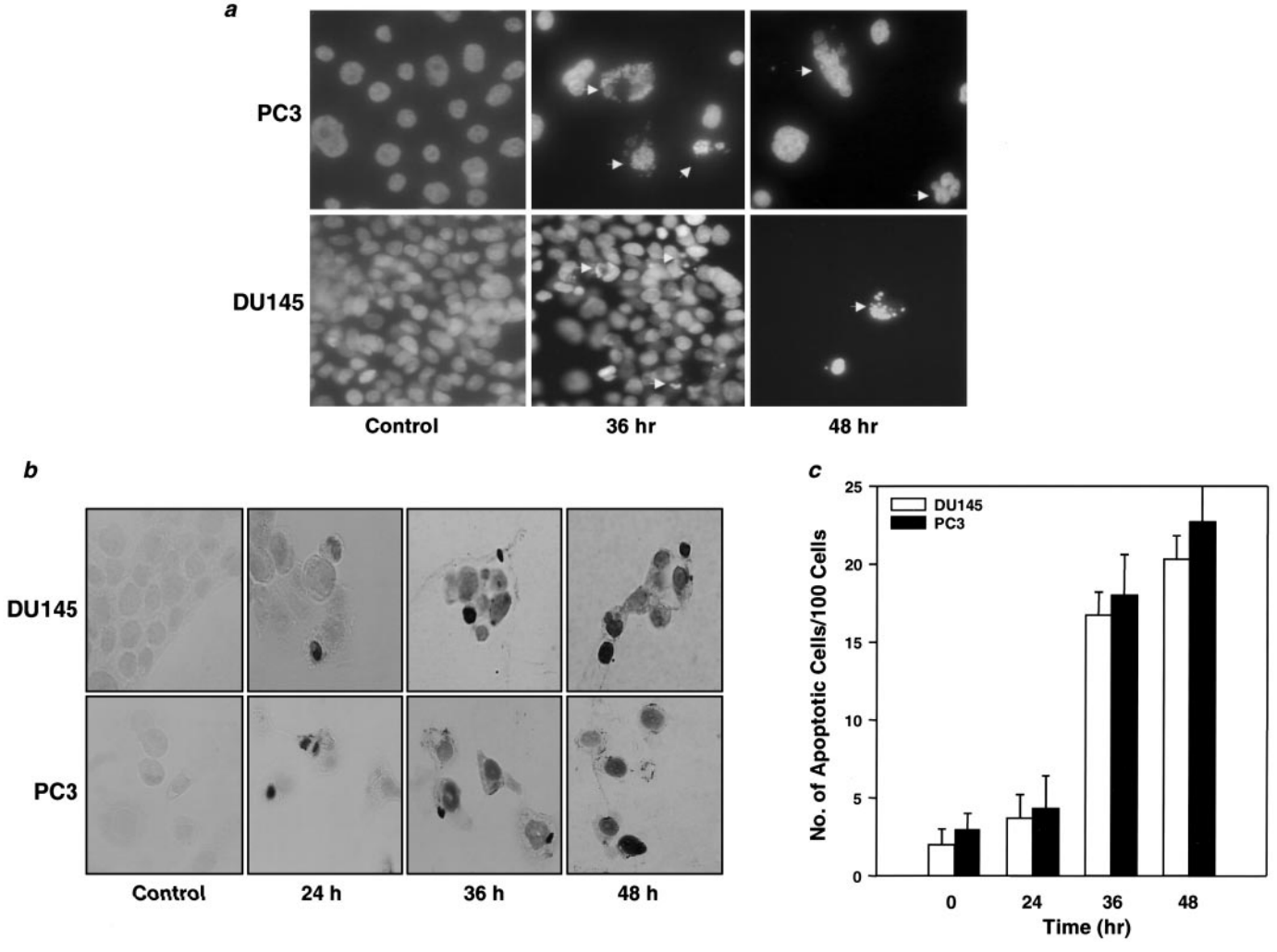

Fig. 3. Raloxifene-induced apoptosis in prostate cancer cells. *a*, photomicrographs of nuclear morphology. *b*, TUNEL apoptotic assay. *c*, number of apoptotic cells per 100 cells. Using the nucleus-specific staining DAPI and TUNEL assay, a time-dependent increase in the rate of nuclear fragmentation and apoptosis was observed in both cell lines after treatment with 10^{-6} M raloxifene.

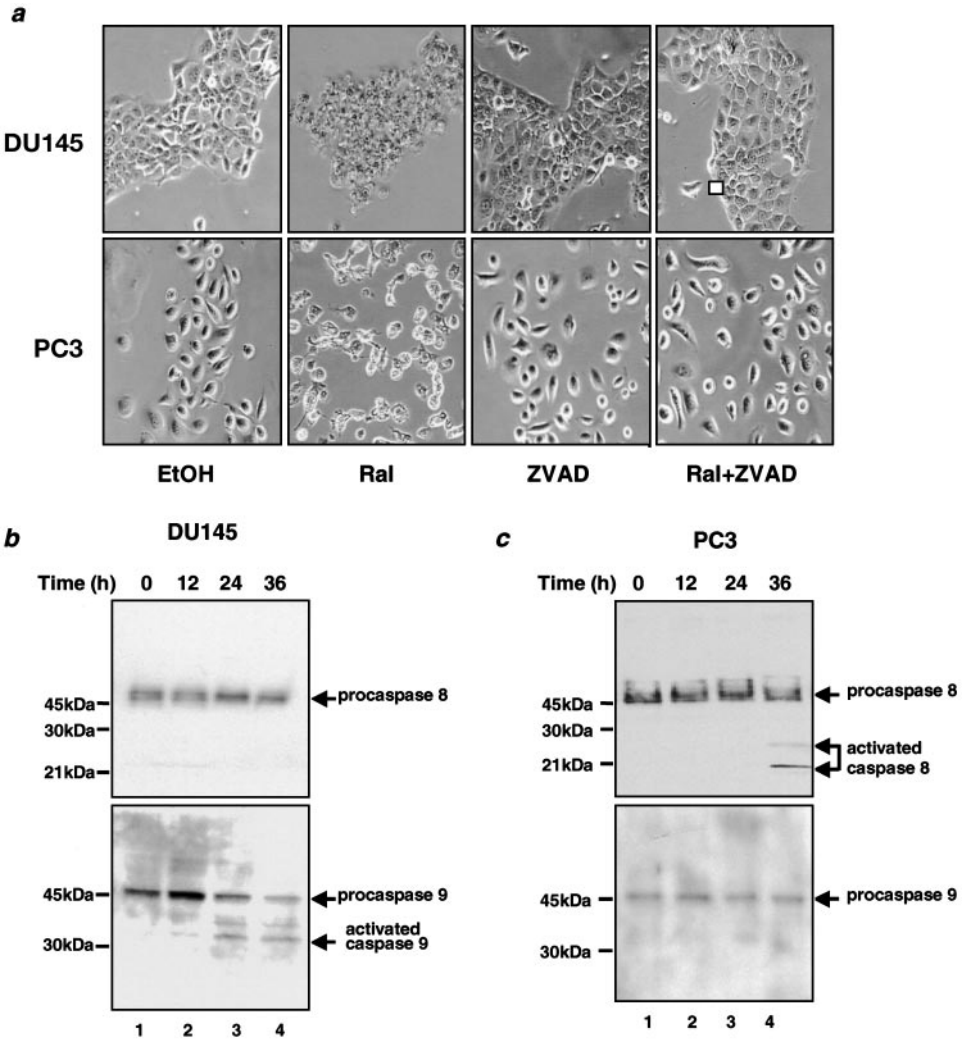

Fig. 4. Caspase in raloxifene-induced apoptosis. *a*, cellular morphology after treatment with the pan-caspase inhibitor ZVAD. *b*, immunoblot for caspases 8 and 9 in DU145 cells. *c*, immunoblot for caspases 8 and 9 in PC3 cells. Pretreatment with ZVAD completely blocked the dramatic change observed previously in cellular morphology induced by raloxifene at 10^{-6} M. Caspase 8 activation was observed in PC3 cells, whereas caspase 9 activation was detected in DU145 cells.

To show that the raloxifene-induced cell death is apoptosis, TUNEL apoptotic assay was carried out (Fig. 3, *b* and *c*). As indicated by the number of dark brown positive cells, there were significant increases in the rate of apoptosis in a time-dependent manner after raloxifene treatment.

Because caspase activation is usually necessary for apoptosis, cells were treated with the pan-caspase inhibitor, ZVAD, before raloxifene treatment. The results revealed that the dramatic change in cellular morphology induced by raloxifene was no longer observed after the addition of ZVAD to the culture medium (Fig. 4*a*). To determine the possibility that caspases 8 and 9 are involved in raloxifene-induced apoptosis, immunoblot analysis was carried out. Raloxifene treatment caused caspase 9 activation in DU145 cells (Fig. 4*b*) and caspase 8 activation in PC3 cells (Fig. 4*c*). These results suggest that the raloxifene-induced cell death in androgen-independent human prostate cancer cell lines is apoptosis and that the actual apoptotic pathway varies with cell types and involves both caspase 8-dependent and –independent pathways.

DISCUSSION

Results of the present study demonstrated that the mixed estrogen agonist/antagonist raloxifene induces cell death in androgen-independent human prostate cancer cell lines. Analysis using nuclear staining, TUNEL apoptotic assay, and immunoblot analysis for caspases 8 and 9 demonstrated that the observed cell death after the raloxifene treatment is apoptosis. 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 patients with hormone refractory prostate cancer.

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 which suggests that estrogens and its receptors are important regulators of the prostate. First, the level of expression of ER-β is highest in the prostate (21). In fact, ER-β was originally cloned from a rat prostate cDNA library (15). Second, ER-β knockout mice have benign prostate hyperplasia (19). Third, Bonkhoff *et al.* (17) demonstrated a preliminary association between increased levels of ER-α expression and 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, it was demonstrated that all three androgen-independent prostate cancer cell lines express ER-β, whereas two of the three cell lines express ER-α. These results are consistent with previous reports in which ER-α was detected in PC3 cells, whereas ER-β was seen in PC3 and DU145 cells (20). The consistent expression of ERs in human prostate cancer cell lines suggests 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 (10, 11), is a mixed estrogen agonist/antagonist. Clinical investigations have demonstrated that it is a safe agent for prevention of both osteoporosis and breast cancer (6, 7). In the present study, it was demonstrated that raloxifene causes cell death in a dose- and time-dependent manner in androgen-insensitive human prostate cancer cell lines. This observed cell death was shown to be apoptosis because nuclear fragmentation was detected in association with a positive TUNEL assay. Furthermore, pretreatment with the pan-caspase inhibitor ZVAD before exposure to raloxifene prevented the dramatic change observed previously in cellular morphology induced by raloxifene. The raloxifene-induced apoptosis in prostate cancer cells may be ER mediated because all three cell lines express ER-β, whereas PC3M and PC3 cells express both ER-α and -β. Because ERs in PC3 cells have been shown to be functional (20), it is likely that these ERs in prostate cancer cell lines are physiologically significant. Alternatively, the presence of high-affinity binding sites for SERMS that are independent of ER binding has been demonstrated in multiple systems (22, 23). Because these ER-independent SERM-binding sites are usually more abundant than ER (22, 23), it is possible that the observed raloxifene-induced apoptosis is mediated through an ER-independent pathway in human prostate cancer cells.

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 (24). The three pathways are similar in that they eventually involve activation of caspases; however, the subtypes of caspases that are activated appear to differ significantly among the varying apoptotic pathways. In the present study, the activation of caspases 8 and 9 has been demonstrated in PC3 and DU145 cells, respectively. Because different caspases are activated in the two prostate cancer cell lines, raloxifene-induced apoptosis likely involves multiple and independent pathways.

Interestingly, the magnitude of growth inhibition induced by raloxifene was significantly different between ER-α-positive (PC3 and PC3M) and ER-α-negative cells (DU145). However, the rate of apoptosis was similar between the two types of cells. The mechanism for this observed difference in growth inhibition remains unclear at the present time. However, the present data suggest that raloxifene affects at least two different signaling pathways, growth inhibition and apoptosis, in prostate cancer cells. Furthermore, our data imply that the expression of ER-α may render prostate cancer cells resistant to the growth inhibitory but not apoptotic effect of raloxifene. Additional work is under way to verify this concept.

Lastly, it should be pointed out that the concentration of raloxifene used in the majority of experiments in this study was 10^{-6} M. Clinical trials that investigated the utility of raloxifene have demonstrated that the serum concentration of raloxifene is in the 10^{-9} M range when given the currently recommended dose of 30–150 mg/day. Thus, it is possible that the 10^{-6} M may not be an achievable concentration *in vivo*. Nevertheless, we believe that raloxifene may be an effective agent in treating androgen-independent prostate cancer patients for the following two reasons: (*a*) the effect of raloxifene was observed initially in prostate cancer cell lines at 10^{-9} M after 4 days of treatment; and (*b*) the profile of sensitivity to raloxifene between breast and prostate cancer cell lines was similar. Currently, we are planning an animal study to determine the maximum achievable serum concentration of raloxifene *in vivo*.

In conclusion, the present study demonstrated that raloxifene, a mixed estrogen agonist/antagonist, induces apoptosis in androgen-independent human prostate cancer cell lines. Because raloxifene has minimal adverse effects while effectively preventing osteoporosis and breast cancer, the present study provides a rationale for a clinical trial in hormone-refractory prostate cancer patients. In the future, the feasibility of raloxifene in treating and preventing prostate cancer will be investigated.

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