Selective estrogen receptor modulator is a proven agent for chemoprevention and chemotherapy of cancer. Raloxifene, a mixed estrogen agonist/antagonist, was developed to prevent osteoporosis and potentially reduce the risk of breast cancer. In this study, we examined the effect of raloxifene on the TSU-PR1 cell line. This cell line was originally reported to be a prostate cancer cell line, but recently it has been shown to be a human bladder transitional cell carcinoma cell line. The TSU-PR1 cell line contains high levels of estrogen receptor β. Following treatment with raloxifene, evidence of apoptosis, including change in nuclear morphology, DNA fragmentation, and cytochrome c release, was observed in a dose-dependent manner in the TSU-PR1 cells (10⁻⁹ to 10⁻⁸ M range). We observed no detectable change in the steady-state levels of Bax, Bcl-2, and Bcl-XL following raloxifene treatment. However, raloxifene induced caspase-dependent cleavage of BAD to generate a 15-kDa truncated protein. Overexpression of a double mutant BAD resistant to caspase 3 cleavage blocked raloxifene-induced apoptosis. These results demonstrate that raloxifene induces apoptosis through the cleavage of BAD in TSU-PR1 cells. This molecular mechanism of apoptosis suggests that raloxifene may be a therapeutic agent for human bladder cancer.

Raloxifene is the prototypical selective estrogen receptor modulator (SERM) that has been shown to prevent osteoporosis and breast cancer (1, 2). Raloxifene binds to both ERα and ERβ with high affinity (3, 4); however, the binding affinity to ERα is four times higher than ERβ. Among the SERMs, raloxifene is unique in that it is an estrogen antagonist in the uterus (5). In the breast and bone, however, all SERMs act as estrogen agonists and antagonists, respectively (6). The mechanism for the observed tissue-specific effect of SERMs is currently unknown.

Two estrogen receptors (ERs) are known to mediate the physiological responses to estrogen, ERα (7) and ERβ (8–10). ERα and ERβ have revealed some overlap of function, but also have significant differences in their ligand-binding and transcriptional properties (11, 12). ERα and ERβ exhibit both differential and overlapping tissue distribution. Whereas ERα has been predominantly found in the anterior pituitary, uterus, vagina, testis, liver, and kidney, ERβ is predominantly expressed in thyroid, ovary, prostate, skin, bladder, lungs, gastrointestinal tract, cartilage, and bone (13, 14). In the epithelium of the urinary bladder of adult rat, high levels of ERβ, but not ERα, were detected (15), suggesting that ERβ plays an important role in the bladder. The finding that older ERβ knock-out mice exhibit prostate and bladder hyperplasia (16) also suggests that the estrogen receptor is a reasonable target for therapeutic intervention in prostate and bladder cancer patients.

Apoptotic stimuli activate caspases through mitochondria-dependent and mitochondria-independent pathways. The Bcl-2 family proteins serve as critical regulators of mitochondria-dependent pathways. Antiapoptotic proteins (Bcl-2 and Bcl-XL) reside in the mitochondria, whereas proapoptotic proteins (BAX, BAD, and BID) reside in the cytosol. Upon apoptotic stimuli, proapoptotic proteins translocate to the mitochondrial membrane, and mitochondria lose their membrane potential and release cytochrome c (17, 18). In IL-3-dependent lymphoid cells, BAD is a key regulator of apoptosis (19). The function of BAD is regulated by reversible phosphorylation and binding of 14-3-3 proteins (20). Deprivation of survival factors induces BAD dephosphorylation by the specific serine/threonine phosphatase PP1α (21), resulting in dissociation of BAD from 14-3-3 proteins and translocation to the mitochondria, where BAD interacts with Bcl-XL and Bcl-2 and antagonizes their anti-apoptotic functions. BAD is cleaved by a caspase(s) at its N terminus to generate a 15-kDa truncated protein following IL-3 deprivation-induced apoptosis in murine myeloid precursor 32Dcl3 cells. The 15-kDa truncated BAD is a more potent inducer of apoptosis than the wild-type BAD, whereas a mutant BAD, resistant to caspase 3 cleavage, is a weaker inducer of apoptosis (22).

In this study, we have investigated the effect of raloxifene on TSU-PR1, a cell line that was originally reported to be a human prostate cancer cell line but has been shown recently (23) to be a bladder cancer cell line. Raloxifene treatment induces apoptosis in these cells and induces caspase-dependent cleavage of BAD. A mutant BAD resistant to caspase 3 cleavage blocked raloxifene-induced apoptosis.

MATERIALS AND METHODS

Cell Culture—Human TSU-PR1 and PC3 M cells were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS), penicillin (100
Raloxifene Induces Apoptosis in Human Cancer Cells

Assessment of Mitochondrial Transmembrane Potential—Changes in mitochondrial membrane potential were determined by staining cells with the fluorochrome, chloromethyl-X-rosamine (CMX-Ros) (Molecular Probes, Eugene, OR). One million cells were incubated with 100 nM CMX-Ros in growth medium for 30 min at 37 °C in the dark and then transferred to a 96-well plate. After 10 min of incubation at 72 °C, the cells were washed twice with phosphate-buffered saline, and cells were cultured for 3 days in phenol-red free RPMI 1640 supplemented with either 10% or 10% charcoal-stripped FBS (cFBS) containing raloxifene at 10−8 M in the presence or absence of estradiol. Raloxifene was added such that the ratio of 10% ethanol to medium was maintained at 10% FBS, and cells were grown for an additional 24 h. The conditioned medium containing recombinant retroviruses was collected and filtered through 0.45-μm polysulfonic filters. Eight ml of these supernatants were applied immediately to TSU-PR1 cells, which had been plated 24 h before infection at a density of 1 × 105 cells/100-mm tissue culture dish and transfected with the pLXSN retrovector (empty or containing HA-WT-BAD, HA-DM56/61 BAD, or tBADHA cDNA), by the calcium phosphate method. Immediately after transfection, cells were treated with 25 μg/mL chloroquine to increase transfection efficiency. Six hours after transfection, the medium was replaced with fresh Dulbecco modified Eagle's medium (DM56/61), or truncated BAD.

After 14 days, individual clones picked from plates of the recombinant retrovirus-infected cells were transferred to 6-microwell plates and expanded to generate cell clones stably expressing WT-type, double mutants (DM56/61), or truncated BAD.

RNA Isolation and RT-PCR—RT-PCR for ERα and ERβ was carried out as described previously (24). Cells were harvested, and total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Once isolated, total RNA was reverse-transcribed using Superscript (Invitrogen) and random hexamer employing the following conditions: 42 °C for 50 min and 70 °C for 15 min. Following 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 35 cycles followed by 10 min of incubation at 72 °C. The following primers were used: ERα, 5′-primer, tacgtcagcatgaacgag, and 3′-primer, C'accctgtgagattgaggaac, and 3′-primer, tgctgaggacatcaagtgg; glyceraldehyde-3-phosphate dehydrogenase, 5′-primer, accaggcagctcagac, and 3′-primer, tcacccaagttggtgac.

The PCR products 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.

TUNEL Assay—WT-BAD, DM56/61-BAD, and t-BAD TSU-PR1 cells were plated at 5 × 104 cells/well in chamber slide (Nalge Nunc, Rochester, NY) and incubated for 24 h prior to treatment with raloxifene as described previously. The medium was replaced with phenol-red free RPMI 1640 containing 1% charcoal-stripped FBS. The cells were treated with 10−7 m raloxifene for 48 h and fixed with 4% paraformaldehyde, pH 7.4, for 10 min. Apoptotic cells were assessed by measuring DNA fragmentation in a standard deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay according to the instructions of the kit (In Situ Cell Death Detection Kit, POD, Roche Molecular Biochemicals, Mannheim, Germany). Immunoblot Analysis—Whole-cell extracts were obtained in a 1% Triton X-100 lysis buffer (50 m M Tris-Cl, pH 8.0, 150 m M sodium chloride, 1 m M EDTA, 2.5 m M sodium pyrophosphate, 1 m M sodium orthovanadate, 1 m M β-glycerophosphate, 1 m M μg/mL leupeptin, and 1 m M phenylmethylsulfonyl fluoride). Western blotting was performed as using anti-Bax (Upstate Biotechnology, Inc.), anti-ERα (Upstate Biotechnology, Inc.), anti-BAD (N-19, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bcl-2 (N-19, Santa Cruz Biotechnology), anti-Bcl-2 (S-18; Santa Cruz Biotechnology), anti-Bax (P-19; Santa Cruz Biotechnology), anti-Bad (M-20; Santa Cruz Biotechnology), anti-cytocrome c (7H8.2C12; BD PharMingen), and anti-HA (C-11; Santa Cruz Biotechnology) antibodies. Protein samples were heated at 95 °C for 5 min and analyzed by SDS-16% PAGE.
ptosis, the steady-state levels of several Bcl-2 family proteins were measured by Western blot analysis. We detected neither significant change in the expression nor cleaved products of Bax, Bcl-2, or Bcl-XL over the raloxifene treatment time course (Fig. 3A). Because commercially available antibody against BAD protein does not recognize the human BAD protein, we generated stable cell lines expressing WT-BAD, DM56/61-BAD, and truncated BAD by infecting cells with retroviruses encoding the HA-tagged WT-BAD, DM56/61, or tBAD68 in order to determine the involvement of BAD in raloxifene-induced apoptosis. Puromycin-resistant colonies were selected, and expression levels of HA-tagged WT-BAD, DM56/61 BAD, or t-BAD68 were examined by Western blotting with an antibody raised against the HA epitope (Fig. 3B). Treatment of WT-BAD-expressing TSU-PR1 cells with raloxifene induced an ~15-kDa cleavage product of BAD as early as 24 h after raloxifene treatment, and cleavage was increased over the raloxifene treatment time course (Fig. 3C).

Effect of Caspase Inhibitor and Cycloheximde on the Cleavage of BAD—It has been shown previously that treatment of 32Dc13 myeloid precursor cells with the general caspase inhibitor Z-VAD-fmk prevents the generation of the 15-kDa cleaved form of BAD (25). Therefore, we assessed the possible involvement of caspases in the cleavage of BAD in TSU-PR1 cells. Pretreatment of TSU-PR1 cells with both raloxifene (10⁻⁷ M) and estradiol (10⁻⁷ M) for 48 h, and then TUNEL assays were performed. The representative of three independent assays is shown. D, TUNEL-positive apoptotic cells were counted, and the percentage of apoptotic cells was graphed.

Fig. 3. Expression of ER and effect of raloxifene on proliferation of a TSU-PR1 cell line. A, RT-PCR showed that a human prostate cancer cell line (PC3 M) and a TSU-PR1 cell line were positive for ERβ, whereas only PC3 M expressed ERα. 10⁻⁷ M raloxifene treatment decreased the expression of ER receptors in both cells. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, Western blot analysis for ER. The molecular weight of ERα recognized by the anti-ERα antibody (from Upstate Biotechnology, Inc.) is about 66 kDa. There was near-complete cell death at 10⁻⁷ M raloxifene in the presence of either 1% cFBS (C) or 10% cFBS (D), suggesting that the observed cytocidal effect of raloxifene at concentration higher than 10⁻⁶ M is likely non-ERα-mediated.
the protein synthesis inhibitor, cycloheximide, for 1 h followed by treatment with raloxifene for 48 h. Pretreatment with cycloheximide inhibited the raloxifene-induced cleavage of BAD in a dose-dependent fashion (Fig. 4B), suggesting that a de novo protein synthesis is required for the generation of the 15-kDa cleaved form of BAD by raloxifene.

**Effects of WT-BAD, DM56/61-BAD, and t-BAD on Raloxifene-induced Apoptosis and Cleavage of BAD**—To compare the effects of DM56/61 and truncated BAD on raloxifene-induced apoptosis, stable cell lines expressing WT-BAD, DM56/61-BAD, and t-BAD were treated with raloxifene for 48 h. The extent of raloxifene-induced apoptosis was estimated by the TUNEL assay. Overexpression of DM56/61-BAD reduced raloxifene-induced apoptosis, whereas overexpression of tBAD\(_{15}\) increased in raloxifene-induced apoptosis, as compared with WT-BAD (Fig. 5, A and B). Also, overexpression of DM56/61-BAD blocked generation of the 15-kDa cleaved form of BAD by raloxifene (Fig. 5C).

**Alteration of Mitochondrial Membrane Potential and the Release of Cytochrome c in Cells Expressing WT-BAD and DM56/61 BAD**—Data suggest that t-BAD regulates mitochondrial membrane potential (25). We examined changes in the mitochondrial membrane potential of cells expressing WT-BAD or DM56/61-BAD using CMX-Ros as a fluorescent probe in flow cytometry. Increased CMX-Ros accumulation reflects an increase in mitochondrial membrane potential. Cells were incubated in the absence or presence of raloxifene for 48 h followed by staining with CMX-Ros for flow cytometry. CMX-Ros fluorescence was reduced in the vector control and WT-BAD-expressing TSU-PR1 cells. However, these changes in mitochondrial membrane potential were not seen in DM56/61-BAD-expressing TSU-PR1 cells (Fig. 6A). Based on these results, we examined whether the loss in mitochondrial membrane potential could be coincident with the release of cytochrome c. After incubation of the cells for 48 h in the absence or presence of 10\(^{-7}\) m raloxifene, mitochondria were separated from the cytosol, and cytochrome c content was analyzed by Western blot. In WT-BAD-expressing TSU-PR1 cells, cytochrome c content decreased markedly in the mitochondria, corresponding to increased levels in the cytosol. However, raloxifene-induced cytochrome c release from mitochondria to cytosol was markedly reduced in DM56/61 BAD-expressing TSU-PR1 cells (Fig. 6B).

**DISCUSSION**

In this study, data suggest that the mixed estrogen agonist/antagonist raloxifene induces apoptosis in a dose- and time-dependent manner in ER\(\beta\)-positive TSU-PR1 through the cleavage of BAD. The cleaved products of BAD, in turn, caused cytochrome c release in TSU-PR1 cells. Because TSU-PR1 cells have been demonstrated recently to be a human bladder transitional cell carcinoma cell line (23), these observations provide a new potential therapeutic target for human bladder cancer.

Raloxifene, an SERM that binds to both ER\(\alpha\) and ER\(\beta\) with high affinity (3, 4), is a mixed estrogen agonist/antagonist. Raloxifene is a safe agent for prevention of both osteoporosis and breast cancer (1, 2). ER\(\beta\), first cloned in the rat prostate, is expressed in various human tissues. ER\(\alpha\) is the predominant isoform in breast and uterine tissue, whereas ER\(\beta\) is expressed in significant quantities in the urogenital tract, the central nervous system, and endothelial cells. ER\(\alpha\) and ER\(\beta\) exhibit differences in binding affinity and potency and might be differentially regulated in estrogen-sensitive tissues (26). In this study, we used TSU-PR1 cells to investigate the effect of raloxifene. TSU-PR1 cells were originally isolated as a human prostate cancer cell line, but recently van Bokhoven et al. (23) demonstrated the cell line to be a human bladder cancer cell line. We observed that TSU-PR1 expresses ER\(\beta\), not ER\(\alpha\).
Therefore, the results of the present study suggest that estrogen/estrogen receptors may be potential targets for therapeutic intervention in bladder cancer patients.

Bcl-2 family proteins are key regulators of apoptosis via heterodimerization between prosurvival Bcl-2 proteins and proapoptotic Bcl-2 proteins (27, 28). Proapoptotic Bcl-2 proteins consist of multidomain members (Bax and Bak) and BH3 domain-only members (BAD, Bid, Bim, and Noxa). A BH-3 domain-only member interacts with a multidomain member upstream of an adaptor and of caspases. BH-3 domain-only members require multidomain Bax or Bak to initiate cytochrome c, Apaf-1-driven caspase activation, and caspase-independent mitochondrial dysfunction (18, 29). BAD is a BH3 only proapoptotic member that shares substantial sequence homology only within the BH3 amphipathic α-helical domain (28, 31). The activity of BAD is regulated by changes in phosphorylation and subcellular localization. It is known that BAD is phosphorylated on one or more of three serine residues, Ser-112, Ser-116,
136, or Ser-155, in response to survival factors, and phosphorylation of these serines sequesters BAD in the cytosol, bound to 14-3-3 proteins (32–33, 35, 36). Recently, it was shown that a protein phosphatase 2A (PP2A) or a PP2A-like phosphatase catalyzes BAD dephosphorylation and regulates its proapoptotic activity in IL-3-dependent lymphoid cells by a mechanism requiring dissociation from 14-3-3 (37). Upon increase in calcium influx or growth factor deprivation, phosphorylated BAD is rapidly dephosphorylated by the specific serine-phosphatase calcineurin (38) or PP1 (39) and translocates to the mitochondrial outer membrane where, through its BH3 domain, it interacts with antiapoptotic Bcl-2 and Bcl-XL (18, 28, 31).

Following IL-3 deprivation of 32Dc13 myeloid precursor cells, BAD is cleaved at its N terminus to generate two smaller products, one very similar in size to the full-length protein (26 kDa) and the second −15 kDa. Transforming growth factor-β1 also induces caspase-dependent cleavage of BAD at its N terminus to generate a 15-kDa truncated protein (40). These results suggest that cleavage of BAD in response to various apoptotic stimuli may be one of the major mechanisms in the process of apoptosis. In TSU-PR1 cells, raloxifene generates only the −15-kDa cleaved form of BAD. In vitro studies have shown that BAD is cleaved by caspases 2, 3, 7, 8, and 10 (22). In this study, generation of the −15-kDa truncated form of BAD was blocked by the caspase inhibitor, Z-VAD-fmk, suggesting that it is the product of caspase activity. Cleavage of BAD enhances sensitivity to apoptosis because raloxifene induces cell death more rapidly in cells expressing truncated BAD than in cells overexpressing wild-type BAD, whereas raloxifene-induced cell death is reduced in cells expressing caspase-resistant mutant BAD.

Proapoptotic Bcl-2 family proteins, including BAD and Bak, induce changes in mitochondrial membrane permeability, often accompanied by cytochrome c release into the cytosol and activation of downstream caspases (28, 39). In contrast, Bcl-XL inhibits cytochrome c release through a physical interaction with components of the voltage-dependent anion channel via a mechanism inhibited by several pro-apoptotic Bcl-2 proteins (30, 41). Recently, it was shown that the transforming growth factor-β1-induced mitochondrial cytochrome c release is inhibited by Bcl-XL (34). Truncated BAD has a similar or higher affinity for Bcl-XL than wild-type BAD, and it is a more potent inducer of cytochrome c release than full-length wild-type BAD. It has been suggested that truncated BAD may be poorly phosphorylated at Ser-155 because phosphorylation of Ser-136 seems to be required for Ser-155 phosphorylation (22). This poor phosphorylation would enhance the interaction of truncated BAD with Bcl-XL. Therefore, increased generation of truncated BAD, induced by raloxifene, may result in enhanced cytochrome c release by antagonizing antiapoptotic Bcl-XL.

In conclusion, we have demonstrated that raloxifene, a mixed estrogen agonist/antagonist, induces apoptosis in TSU-PR1 cells. Cleavage of BAD, leading to the cytochrome c release, serves as a novel mechanism of raloxifene-induced apoptosis. These results provide valuable insight concerning the role of estrogen/estrogen receptor in bladder cancer cells. Because raloxifene has minimal side effects while effectively preventing osteoporosis and breast cancer, this study suggests raloxifene as a potential treatment in patients with advanced bladder cancer.

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