Induction of Apoptosis by Bexarotene in Cutaneous T-Cell Lymphoma Cells: Relevance to Mechanism of Therapeutic Action

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ABSTRACT

Purpose: Bexarotene is the first synthetic rexinoid approved for the treatment of all stages of cutaneous T-cell lymphoma (CTCL) however the mechanism of bexarotene action is unknown. We examined the effects of bexarotene on induction of apoptosis and expression of its cognate receptors in well-established CTCL cell lines (MI, Hut78, and HH).

Experimental Design: CTCL cells were treated with 0.1, 1, and 10 μM bexarotene for 24, 48, 72, and 96 h. Apoptosis was determined by flow-cytometry analysis of sub-G1 hypodiploid nuclei and annexin V binding populations. Apoptosis-associated proteins and retinoid receptors were detected by Western blots.

Results: Bexarotene treatment at 1 and 10 μM for 96 h increased the number of cells with sub-G1 populations and annexin V binding in a dose-dependent manner compared with vehicle controls (DMSO) in all three cell lines, respectively. Bexarotene treatment suppressed the expression of retinoid X receptor α and retinoic acid receptor α proteins in all three lines compared with untreated controls. Bexarotene treatment decreased the protein levels of survivin, activated caspase-3, and cleaved poly(ADP-Ribose) polymerase, as well as down-regulation of poly(ADP-Ribose) polymerase, as well as down-regulation of retinoid X receptor α, retinoic acid receptor α, and survivin. These findings support apoptosis as a mechanism for bexarotene therapy in CTCL.

INTRODUCTION

CTCL is a group of lymphoproliferative disorders characterized by localization of malignant T lymphocytes to the skin at presentation. MF, the most common and indolent form of CTCL, is characterized by patches, plaques, or tumors containing epidermotropic CD4+CD45RO+ helper/memory T cells. MF may evolve into a leukemic variant, SS, or transform to large cell lymphoma (1, 2). The pathogenesis of CTCL remains largely unknown. The clinical evolution of CTCL is strikingly slow in most cases, especially when the involved area is <10% of the total cutaneous surface, and the overall rate of disease-related death is low except for tumor stage and erythrodermic patients (3). This outcome is reminiscent of nodal follicular B-cell lymphoma, in which defective apoptosis, with subsequent accumulation of B cells by overexpression of the antiapoptotic bcl-2 protein, is now considered a main etiological factor (4). Together with the observation that the proliferative index is usually low in CTCL, this similarity prompted us to hypothesize that CTCL might also be a disorder of accumulation, rather than purely malignant proliferation of CD4+ memory T cells. Dysregulated apoptosis of skin-homing memory T cells may be involved in pathogenesis and therapeutic implications of CTCL.

Retinoids, such as all-trans-retinoic acid and 13-cis-retinoic acid and synthetic analogues (acitretin, etretinate, and isotretinoin) have long been used alone and in combination with other therapies for CTCL (5–11). Bexarotene is the first synthetic highly selective RXR retinoid (a rexinoid) to be studied in humans (12). Bexarotene was recently approved by the Food and Drug Administration in both oral capsule and topical gel formulations for the treatment of cutaneous manifestations of CTCL (13). Clinical trials have shown that bexarotene is safe and effective for the treatment of all stages of CTCL in patients refractory to at least one systemic therapy (14–17). In addition, topical bexarotene is also effective in clearing CTCL skin lesions (15, 18).

Retinoids are potent biological modulators that exert their effects through two subfamilies of intracellular receptors: RARs and RXRs. Each subfamily has three subtypes of tissue specific proteins.
receptor isoforms, designated as α, β, and γ. Each receptor subtype is thought to control both unique and overlapping target genes (19). Bexarotene inhibits cell growth and/or induces apoptosis in human HL-60 promyelocytic leukemia and epithelial cancer cells (20–23). Bexarotene’s mechanism of action in CTCL has not yet been demonstrated. In this study, we examined the effects of bexarotene on cell growth, apoptosis, and expression of apoptosis-related proteins and retinoid receptors in well-established CTCL cell lines (MJ, Hut78, and HH).

MATERIALS AND METHODS

Cell Lines and Cell Culture. Human MJ, Hut78, and HH CTCL cell lines, derived from peripheral blood of patients with MFs, SS, and non-MF/SS aggressive CTCL respectively (24–26), were obtained from American Type Culture Collection (Rockville, MD). They were maintained in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, Utah), 2 mM HEPES, and 1% penicillin-streptomycin in a humidified atmosphere with 5% CO₂ at 37°C.

Bexarotene Treatment. Bexarotene [LGD1069; 4-[1-(3, 5, 5, 8, 8-pentamethyl-5, 6, 7, 8-terahydro-2-naphthyl)ethenyl]-benzoic acid] was obtained from Ligand Pharmaceuticals Inc. (San Diego, CA). A stock solution of bexarotene at the concentration of 10 mM was prepared in DMSO (DMSO) and stored at −20°C in the dark. The stock solution was added directly to RPMI 1640 to final working concentrations of 0.1, 1, and 10 µM. An equal volume of DMSO was added to the medium for the control experiments. MJ, Hut78, and HH cells (1 × 10⁵ cells/ml) were incubated with 0.1, 1, and 10 µM bexarotene for 24, 48, 72, and 96 h. To avoid possible effects of cell density on cell growth and survival, fresh medium and corresponding concentrations of bexarotene were added at 48 h. Camptothecin (Sigma Chemical Co.) was used as a positive control for inhibition of cell growth and induction of apoptosis.

Cell Viability Assay. Cell viability was measured by the CellTiter 96 AQeuous One Solution Assay (Promega, Madison, WI) according to the manufacturer’s instructions. This assay is a nonradioactive procedure that measures metabolic function that directly correlates with living cell numbers (27). Aliquots of 1 × 10⁴ cells/well were distributed in 96-well microplates (BD Biosciences, Bedford, MA) in 100 µl of medium and incubated with 0.1, 1, and 10 µM bexarotene for 24, 48, 72, and 96 h. Then 20 µl of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt were added to each well and incubated for an additional 4 h. The relative cell viability was determined by ELISA with a 490-nm filter. Each experiment was performed in triplicate and repeated a minimum of three times.

Flow Cytometry Analysis of Cell Cycle. MJ, Hut78, and HH cells (1 × 10⁵ cells/ml) were incubated with or without 0.1, 1, and 10 µM bexarotene for 24, 48, 72, and 96 h. Cells were collected, washed with cold PBS, fixed in cold (−20°C) 100% ethanol, treated with DNase-free RNase, and stained with 50 µg/ml PI. Distribution of the cell-cycle phase with different DNA contents was determined with a FACSscan flow cytometer (Becton Dickinson, San Jose, CA). In each sample, 10,000 gated events were acquired. Analysis of cell-cycle distribution (including sub-G₁; apoptosis) was performed with Modfit software (Becton Dickinson).

Annexin V Binding Staining. The analysis of annexin V binding was carried out with the Annexin V-FITC Detection Kit I (PharMingen, San Diego, CA) according to the manufacturer’s instructions. Briefly, cells were incubated with or without 0.1, 1, and 10 µM bexarotene for 24, 48, 72, and 96 h. Cells were collected, washed twice with cold PBS, centrifuged at 1500 rpm for 5 min, and resuspended in 1 × binding buffer at a concentration of 10⁶ cells/ml. Then 100 µl of the solution (10⁶ cells/ml) were transferred to a 5-ml culture tube; 5 µl of annexin V-FITC and 5 µl of PI were added. Cells were gently vortex-mixed, and incubated for 15 min at room temperature in the dark. Furthermore, 400 µl of 1 × binding buffer were added to each tube, and samples were analyzed by FACSscan flow cytometry (Becton Dickinson). For each sample, 10,000 gated events were acquired. Annexin V⁺ PI⁻ cells represented the early apoptotic populations. Annexin V⁺ PI⁺ cells represented either late apoptotic or secondary necrotic populations.

Isolation of Cytoplasmic and Nuclear Extracts. Cells (5 × 10⁶) were washed with ice-cold PBS, harvested into 1 ml of PBS, pelleted in a 1.5-ml microcentrifuge tube, and suspended in 400 µl of buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 X protease inhibitor cocktail “complete mini” (Roche, Indianapolis, IN)]. After a 20-min incubation on ice, the mixture was treated with

Fig. 1  Bexarotene treatment inhibits cell growth of CTCL cells. Cells were treated with or without 0.1, 1, and 10 µM bexarotene for 24, 48, 72, and 96 h. Cell viability was determined by comparing vehicle (DMSO) to treated groups with the CellTiter 96 AQeuous One Solution Assay performed in triplicate. Each point represents mean ± SD of triplicate determinations. *, significantly different from vehicle control values (P < 0.05; n = 6).
a 24-gauge syringe five times and then centrifuged briefly to obtain the cytoplasmic supernatant. The nuclear pellet was resuspended in 40–80 μl of buffer C [10 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 1× protease inhibitor cocktail “complete mini”] and incubated at 4°C with shaking for 15 min. Protein concentrations were determined by the Bradford dye-binding protein assay (Bio-Rad, Richmond, CA) with BSA as a standard.

**Western Blot Analysis.** Cytoplasmic (20 μg) or nuclear (10 μg) proteins were subjected to SDS-polyacrylamide gel (8–12%) electrophoresis (SDS-PAGE) followed by electrotransferring onto nitrocellulose membranes (Schleicher & Schuell, Dasse, Germany). The membranes were blocked in 3% powdered milk in TBST [50 mM Tris (pH 7.5), 150 mM NaCl, 0.05% Tween 20], incubated with primary antibody overnight at 4°C with shaking for 15 min. Protein concentrations were determined by the Bradford dye-binding protein assay (Bio-Rad, Richmond, CA) with BSA as a standard.

**Statistical Analysis.** All experiments were performed triplicate unless otherwise noted. Results were expressed as mean ± SD. Statistical significance (P < 0.05) was assessed by the Student’s t test.

**RESULTS**

**Effect of Bexarotene on Cell Viability in CTCL Cells.** To determine whether bexarotene inhibited cell growth of CTCL cells, MJ, Hut78, and HH cell lines were treated with or without 0.1, 1, and 10 μM bexarotene for 24, 48, 72, and 96 h. Their viability was evaluated by the CellTiter 96 AQueous One Solution Assay. As the dose of bexarotene increased from 1 to 10 μM for 96 h, cell growth inhibition was significantly (P < 0.05; n = 6) increased from 10.3% to 26.3%, 15.8% to 35.4%, and 18.2% to 40.8% in MJ, Hut78, and HH compared with vehicle (DMSO), respectively. There was no obvious growth inhibition at the dose of 0.1 to 10 μM over the course of 24 to 72 h in all three lines (Fig. 1).

**Fig. 2 Bexarotene treatment increases sub-G1 populations in CTCL cells.** Cells were treated with or without 0.1, 1, and 10 μM bexarotene for 24, 48, 72, and 96 h. Cell cycle was analyzed by PI-staining and flow cytometry. A, each point represents the percent of sub-G1 populations with hypodiploid DNA content. B, histograms for cell cycle are from analysis of cells treated with 10 μM bexarotene for 96 h. Results are representative of one of three independent experiments.
Effect of Bexarotene on Cell Cycle and Apoptosis in CTCL Cells. To determine whether growth inhibition of bexarotene was attributable to cell-cycle arrest and/or apoptosis in CTCL cells, MJ, Hut78, and HH cell lines were treated with or without 0.1, 1, and 10 μM bexarotene for 24, 48, 72, and 96 h. Annexin V binding was carried out with the Annexin V-FITC Detection Kit. A, each point represents the percentage of both annexin V+PI− and annexin V+PI+ cells. B, annexin V/PI staining was analyzed from cells treated with 10 μM bexarotene for 96 h. The lower right quadrant shows annexin V+PI+ cells. The upper right quadrant represents annexin V+PI− cells. Results are representative of one of three independent experiments.

Effect of Bexarotene on Expression of Apoptosis-associated Proteins in CTCL Cells. To understand the mechanism of bexarotene-induced apoptosis in CTCL cells, Fas/FasL, bcl-2, survivin, caspase-3, and PARP were detected by Western blots in MJ, Hut78, and HH cell lines treated with or without 10 μM bexarotene for 96 h. Our results showed that the protein levels of survivin, but not bcl-2, were decreased by 40%, 30%, and 60% from vehicle (DMSO) levels in MJ, Hut78, and HH cells, respectively. Caspase-3 was activated as indicated by the appearance of active 17-kDa fragments, and PARP was cleaved as shown by the appearance of a signature 85-kDa fragment in all three cell lines (Fig. 4).

Effect of Bexarotene on Expression of Retinoid Receptors in CTCL Cells. To understand how bexarotene regulates the nuclear retinoid receptors, expression of RXR-α and RAR-α was detected by Western blots from proteins extracted from MJ, Hut78, and HH cell lines treated with 10 μM bexarotene for 0, 24, 48, and 72 h. The protein levels of RXR-α and RAR-α were decreased from 20% to 70% and 33% to 66% in all three lines compared with untreated controls, respectively (Fig. 5). These results suggest that bexarotene treatment at clinically relevant concentrations down-regulates expression of RXR-α and RAR-α proteins in all three CTCL cell lines.
DISCUSSION

Apoptosis is a genetically regulated form of cell death characterized by morphological and biochemical changes very different from necrosis (30). Apoptosis plays an important role in embryogenesis, aging, and many diseases including cancer (31). The antitumor action of many chemotherapeutic agents has been attributed to the induction of apoptosis (32). In CTCL, phototherapy and photopheresis work through induction of T-cell apoptosis (33, 34). In this study, we show for the first time that bexarotene at 1 and 10 \( \mu \)M causes apoptosis of CTCL cell lines as determined by the increase of both sub-G1 hypodiploid nuclei and annexin-V-binding populations. In clinical trials, the peak plasma levels of bexarotene are 3.4, 8.3, and 17.5 \( \mu \)M at doses of 300, 400, and 800 mg/m²/day, respectively (14, 16, 35). Thus, the concentrations of bexarotene-induced apoptosis in these CTCL cell lines are consistent with values achieved clinically and suggest that this is a clinically relevant mechanism of action.

Apoptosis is a multistep process and an increasing number of genes have been identified that are involved in the control or execution of apoptosis (36). Caspases play a crucial role in apoptosis. Among the 14 known members of interleukin-1-converting enzyme family of proteases, caspase-3 has been shown to be a key component of the apoptotic machinery (37). Caspase-3 is activated in apoptotic cells and cleaves several cellular proteins, including PARP. The cleavage of PARP is used as a hallmark of apoptosis by various antitumor agents (38). In this study, we show for the first time that bexarotene treatment activates caspase-3 and cleaves PARP in three CTCL cell lines. This implies that activation of caspase-3 is involved in bexarotene-induced apoptosis of CTCL cell lines.

Fas/APO-1 (CD95) is a member of the tumor necrosis factor/nerve growth factor receptor superfamily of cell-surface molecules that transmit apoptosis signals initiated by FasL (39). Fas/FasL pair plays a manifold role in regulating life and death of T lymphocytes (40). Fas is expressed in activated lymphocytes, but its expression is decreased or lost in malignant CTCL cells. Impaired Fas/FasL apoptotic pathway may contribute to pathogenesis and therapeutic implications of CTCL (41, 42). In this study, we are the first to report that CTCL cell lines are devoid of Fas protein and that bexarotene does not restore Fas expression. Thus, loss of Fas cannot prevent bexarotene-induced apoptosis in CTCL cells, suggesting that an apo-
apoptotic pathway independent of Fas/FasL interaction may be involved in bexarotene-induced apoptosis in CTCL cells.

The antiapoptotic protein, bcl-2, has been demonstrated to play an important role in the regulation of apoptosis and to inhibit the induction of apoptosis in lymphocytes by a variety of signals (43). Expression of bcl-2 has been found in CTCL cells and is supposed to increase the survival and the resistance of CTCL cells against radiotherapy and extracorporeal photopheresis (44–46). Recent studies have shown that bcl-2 plays a critical role in controlling the activation of caspases by regulating release of cytochrome c from mitochondria. This indicates that bcl-2 functions upstream of the caspase activation step in the cell death pathway (43, 47). In this study, we show that bexarotene does not obviously change the levels of bcl-2 protein, suggesting that activation of caspase-3 may be independent of bcl-2 action in bexarotene-induced apoptosis.

Survivin is a recently cloned member of the inhibitor of apoptosis protein family (48). Survivin is not expressed in normal adult tissues but is abundantly expressed in fetal tissues, transformed cell types and a variety of human tumors including highly malignant non-Hodgkin’s lymphoma (48, 49). In vitro, survivin is not expressed in resting T cells but expressed in activated T lymphocytes and its expression correlates with apoptosis resistance after lymphocyte activation (50). Survivin suppresses caspase activity and protects cells from apoptosis induced by a variety of agents (51). Our results show for the first time that CTCL cell lines express survivin and bexarotene treatment decreases the protein levels of survivin, suggesting that down-regulation of survivin may be involved in activation of caspase-3 in bexarotene-induced apoptosis.

RXRs function as homodimers or as heterodimers with various receptor partners such as RARs, vitamin D receptors, thyroid receptor, peroxisome proliferator activator receptors, and other orphan receptors. Once activated, these receptors function as transcription factors that regulate the expression of genes that control cellular differentiation, proliferation and apoptosis (52). It has been shown that LG100268 is the most potent RXR selective ligand without any RXR activity (53). TTNPB is a highly selective RXR ligand (54). Neither LG100268 nor TTNPB alone could cause apoptosis. However, LG100268 in combination with TTNPB causes apoptosis in leukemia cells (53). LGD1096 (bexarotene) is less potent than LG100268 in RXR binding and cotransfection experiments and displays some RXR cross-reactivity at higher concentrations (>1 μM) (12, 21, 53). In this study, we show that bexarotene only at 1 μM and 10 μM but not 0.1 μM caused apoptosis in association with down-regulation of RXR-α as well as RAR-α proteins in CTCL cells. These findings suggest that there may be both RXR and RAR activations, and that RARs might be required for bexarotene-induced apoptosis of CTCL cells. Further study is needed to understand how bexarotene down-regulates RXR-α and RAR-α proteins, and to evaluate combinations of different receptor selective ligands in inducing apoptosis of CTCL cells.

In conclusion, we have demonstrated that bexarotene treatment causes apoptosis of CTCL cell lines in association with down-regulation of RXR-α, RAR-α and survivin, activation of caspase-3, and cleavage of PARP. These molecular effects have important implications for understanding how bexarotene therapy affects CTCL in patients. More mechanistic studies are needed to identify new target genes and clarify the molecular details of bexarotene regulated apoptosis that could be used as surrogate end points to analyze other agents and retinoids in clinical development.

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