Biological Effects of Bexarotene in Cutaneous T-Cell Lymphoma

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Objective: To determine the effects of bexarotene on malignant T cells isolated from the peripheral blood of patients with the leukemic variant of cutaneous T-cell lymphoma (Sézary syndrome).

Design, Setting, and Participants: Peripheral blood mononuclear cells from 9 patients with Sézary syndrome and a high burden of circulating malignant T cells (>50% of peripheral blood mononuclear cells) and 6 healthy volunteers underwent evaluation at a university medical center, to test the effects of bexarotene on T cells.

Main Outcome Measures: The capacity of bexarotene to induce apoptosis and its effects on T-cell cytokine production from peripheral blood lymphocytes isolated from patients with Sézary syndrome.

Results: Bexarotene produced dose-dependent apoptosis of peripheral blood T cells from patients with Sézary syndrome. The T cells from approximately two thirds of patients were consistently sensitive to bexarotene, whereas those from the remaining one third of patients were consistently resistant to the apoptotic effects of bexarotene. Bexarotene inhibited mitogen-induced interleukin 4 production by the peripheral blood cells of patients with Sézary syndrome, and this effect correlated with sensitivity of patients’ cells to apoptosis. In contrast to the retinoic acid receptor–specific retinoid, all-trans retinoic acid, bexarotene does not induce the augmentation of interferon γ production.

Conclusions: Bexarotene induces apoptosis of malignant T cells from patients with Sézary syndrome, but the cells from a proportion of patients are resistant to the apoptotic effects. Interleukin 4 production, which can play a role in the systemic immunosuppression that characterizes advancing Sézary syndrome, may be inhibited by bexarotene.

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BEXAROTENE (TARGRETIN) IS A novel third-generation retinoid X receptor (RXR)–selective retinoid approved for the therapy of cutaneous T-cell lymphoma (CTCL). Treatment with bexarotene alone results in a 50% response rate in early and advanced stages of CTCL. The mechanism of action of this agent is not completely understood. A recent study reported the induction of apoptosis in CTCL cell lines (MJ, Hut78, and HH) in association with activation of caspase-3 and cleavage of poly–adenosine diphosphate-ribose polymerase.3 The purpose of this study was to examine a number of putative mechanisms of action directly on malignant T cells derived from patients with Sézary syndrome (hereafter referred to as SS patients), including the in vitro effects of bexarotene on apoptosis and T-cell cytokine production. The combined effects of bexarotene and interferon α-2a (IFN-α) on the induction of apoptosis and ability of bexarotene vs all-trans retinoic acid (ATRA) to induce interferon γ (IFN-γ) production were also evaluated.
PATIENTS

Sézary syndrome was diagnosed on the basis of clinical, histopathological, and immunohistological criteria. All patients met the criteria defined by Vonderheid et al. and demonstrated generalized erythroderma, greater than 20% circulating Sézary cells, and loss of selected pan-T-cell markers by means of flow cytometry. One-micrometer sections of formalin-fixed peripheral blood buffy coats were examined by means of light microscopy for detection of lymphocytes with the cerebriform nuclear morphology typical of Sézary cells. Patients with microscopically characterized erythroderma, greater than 20% circulating Sézary cells, were examined by means of light microscopy. One-micrometer sections of formalin-fixed peripheral blood buffy coats were examined by means of light microscopy for detection of lymphocytes with the cerebriform nuclear morphology typical of Sézary cells. Patients with median tumor burden were defined as those having 20% to 50% circulating Sézary cells; those with high tumor burden, as those having more than 50% circulating Sézary cells. None of the patients had received treatment with bexarotene; all had been treated with extracorporeal photopheresis approximately every 4 weeks. Blood samples from age-matched healthy donors were used as control samples. Donation of blood by patients and healthy donors conformed to the protocols approved by the University of Pennsylvania, Philadelphia, institutional review board, and informed consent was obtained.

MONONUCLEAR CELLS

Phenotypic and functional analyses of mononuclear cells in SS patients and healthy donors were performed on viable frozen peripheral blood mononuclear cells (PBMCs). The PBMCs were collected from blood as previously described, divided into aliquots, and stored in liquid nitrogen. Cells were thawed on the day of the experiment and transferred to culture medium (RPMI 1640) supplemented with 10% fetal bovine serum and a combination of penicillin and streptomycin. Cells were then divided and cultured for detection of apoptosis among treatment groups or under conditions for stimulation of cytokine production. Freshly isolated cells were available from 4 patients and were used in the apoptosis assays. The results were compared with frozen cells from the same patients as a control for the possible effects of freezing.

APOTOPSIS ASSAYS

The PBMCs from the 9 patients with high tumor burden and the 6 healthy volunteers were precultured in 24-well plates at a density of 2 × 10⁶/mL per well in the presence of 50 U of human recombinant interleukin 2 (IL-2; Roche Diagnostics, Indianapolis, Ind) at 37°C. We used IL-2 to increase cell survival in vitro, which resulted in lower baseline apoptosis in all treatment groups when compared with cell cultures without the addition of IL-2. After 12 hours, the cells were harvested, washed twice with phosphate-buffered saline (Gibco BRL) and treated with bexarotene at 1µM and 10µM concentrations. Interferon α at final concentrations of 100 and 1000 U/mL were also added to media alone and combined with 10µM bexarotene in 4 of the patient samples. Camptothecin (Sigma-Aldrich Corp) was used as a positive control for induction of apoptosis. All experiments were performed independently and in triplicate using PBMCs from the same patient. Experiments were also conducted on freshly isolated cells (not subject to freezing) from 4 patients and compared with frozen samples from the same patients. Subsequent to treatment, the PBMCs were incubated at 37°C for 48, 72, and 96 hours. To evaluate the effect of the treatments on in vitro apoptosis, a modification of the terminal deoxynucleotidyl transferase (dUTP) nick-end labeling assay, as previously described, was used. Apoptotic cells exhibit intranucleosomal DNA strand breaks, which generate a multitudes of approximately 180–base pair fragments. These fragments may be subject to an end-labeling reaction, mediated by the enzyme terminal deoxynucleotidyl transferase, using a tagged nucleotide substrate. To identify apoptotic cells, a terminal transferase enzyme derived from calf thymus (Roche Molecular Biochemicals, Mannheim, Germany) and cyanine 5–tagged dUTP (Cy5-dUTP) (Amersham Pharmacia Biotech, Piscataway, NJ) were used to generate homopolymer dUTP tails at the 3’ end of the DNA fragments. Apoptotic cells were identified using a flow cytometer (FACScan; Becton Dickinson, Franklin Lanes, NJ). The percentage of apoptotic cells was calculated by means of the following equation:

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\text{(No. of Cells Incorporating Cy5-dUTP)} \times 100)
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/ \text{Total No. of Cells}

Apoptotic cells were distinguished from necrotic cells on the basis of their size and ability to incorporate the Cy5-dUTP label. Necrotic cells exhibit a loss in cell membrane integrity, characterized by a reduction in their size. This is reflected by a decrease in their forward light-scattering properties. Apoptotic cells retain their membrane integrity, and thus do not exhibit a significant shift in their forward light-scattering properties.

FLOW CYTOMETRIC ANALYSIS

To detect CD4+ subsets of PBMCs, 10⁶ PBMCs per sample were resuspended in phosphate-buffered saline solution with 5% fetal bovine serum and stained with anti-CD4 peridinin chlorophyll protein (BD Biosciences, San Jose, Calif). Murine immunoglobulin of an appropriate isotype was used as a control. Cells were incubated with antibodies for 30 minutes on ice in the dark, then washed, fixed in 2% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, Pa), and permeabilized in 0.1% Triton X surfactant (Roche Diagnostics) in preparation for the terminal dUTP nick-end labeling assay. We acquired 20000 events with a flow cytometer (FACScalibur; Becton Dickinson). Evaluation of the lymphocyte population was performed by gating on CD4+ cells with lymphocyte morphology on the basis of forward and side light-scatter properties. The data were collected and analyzed with the aid of CellQuest software (Becton Dickinson).

CYTOKINE ASSAYS

Interleukin 4

To induce cytokine production, PBMCs from 11 patients with high tumor burden and 5 healthy donors were cultured at 37°C in 24-well plates at a density of 2 × 10⁶/mL per well with or without 10µM bexarotene for 48 hours followed by the addition of 2-ng/µL phytomagglutinin (Inviron Technology, Carlsbad, Calif) for an additional 48 hours. Cell-free supernatants were collected after a total of 96 hours of incubation and frozen at -80°C until analysis. Interleukin 4 production was assayed using an enzyme-linked immunosorbent assay kit (R&D Systems) according to the manufacturer’s recommendations. The sensitivity of this assay was 10 pg/mL. All experiments were performed independently and in triplicate using the same patients’ cells.

Interferon γ

The PBMCs from 6 patients with medium tumor burden and 6 healthy donors were cultured at 37°C in 24-well plates at a density of 2 × 10⁶/mL per well with media, 50 U of IL-2, 10µM bexarotene, 1-ng/mL ATRA, and 10µM bexarotene or 1-ng/mL ATRA in the presence of 50 U of IL-2. A combination of 50 U of IL-2 and 100 U of human recombinant IL-12 (Genetics Institute, Cambridge, Mass) was used as a positive control for IFN-γ production. All treatments were added at time 0, and cell-free supernatants were collected after 72 hours of incubation and stored at
−80°C until analysis. Production of IFN-γ was assayed using an enzyme-linked immunosorbent assay kit (R&D Systems) according to the manufacturer’s recommendations. The sensitivity of this assay was 8 pg/mL. All experiments were performed independently and in triplicate using the same patients’ cells.

RESULTS

APOPTOSIS OF MALIGNANT T CELLS

To determine whether bexarotene causes apoptosis of malignant T cells derived directly from the peripheral blood of SS patients, PBMCs from 9 patients with high tumor burden were treated with and without 1µM and 10µM bexarotene for 72 hours. Apoptotic CD4+ T cells were identified by flow cytometry using a terminal deoxyuridine triphosphate (dUTP) nick-end labeling assay. A, Control sample (media). B, 1µM bexarotene. C, 10µM bexarotene. Values in the upper right quadrant reflect percentage of apoptosis among CD4+ gated cells. Results are representative of 1 of 3 independent experiments. PerCP indicates peridinin chlorophyll protein; cy 5-, cyanine 5–tagged.

Figure 1. Bexarotene induces apoptosis of CD4+ T cells from patients with cutaneous T-cell lymphoma and sensitive high tumor burden. Peripheral blood mononuclear cells were treated with or without 1µM and 10µM bexarotene for 72 hours. Apoptotic CD4+ T cells were identified by flow cytometry using a terminal deoxyuridine triphosphate (dUTP) nick-end labeling assay. A, Control sample (media). B, 1µM bexarotene. C, 10µM bexarotene. Values in the upper right quadrant reflect percentage of apoptosis among CD4+ gated cells. Results are representative of 1 of 3 independent experiments. PerCP indicates peridinin chlorophyll protein; cy 5-, cyanine 5–tagged.

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patients’ lymphocytes and demonstrated consistent findings with respect to sensitivity or resistance to apoptosis after bexarotene treatment. Thus, patients’ cells could be defined as sensitive or resistant to bexarotene with regard to its apoptotic effects. The PBMCs from 6 healthy volunteers were also treated with and without 1µM and 10µM bexarotene for 48, 72, and 96 hours. No apoptosis was evident above that of baseline.

**SYNERGISTIC APOPTOTIC Effects OF BEXAROTENE AND IFN-α ON MALIGNANT T CELLS**

Clinical observations suggest that bexarotene and IFN-α may have synergistic therapeutic effects when used together. Thus, we performed assays to determine whether the clinical synergism could be explained by the synergistic induction of apoptosis in vitro. The addition of IFN-α at concentrations of 100 and 1000 U/mL was evaluated in the samples of 4 patients with high tumor burden previously demonstrated to be sensitive to apoptosis after 72 hours of treatment with bexarotene. At both concentrations, IFN-α consistently induced apoptosis, with an increase of 9% to 43% (average, 23%) compared with media alone. No additive or synergistic effect was observed with the combination of 10µM bexarotene and either concentration of IFN-α.

**BEXAROTENE SUPPRESSION OF IL-4 PRODUCTION BY PBMCs FROM PATIENTS WITH HIGH TUMOR BURDEN**

Cutaneous T-cell lymphoma is typically a proliferation of malignant type 2 helper T cells (T H2) and has been associated with excess IL-4 production. Therefore, the effect of bexarotene on IL-4 production by the malignant T cells of patients with high tumor burden was evaluated. In 5 (45%) of 11 samples, stimulation of PBMCs from patients with high tumor burden and 10µM bexarotene treatment resulted in a 50% reduction in IL-4 levels compared with controls (media alone) (Figure 4). Among the remaining 6 samples, IL-4 production increased 2- to 3-fold in 3 (27%) and was not substantially altered in 3 (27%) by the addition of bexarotene to the cultures. The PBMCs from 5 healthy volunteers were also evaluated. In 4 (80%) of these, the addition of 10µM bexarotene to the cultures resulted in a 1.5- to 5-fold increase in IL-4 production. In 1 (20%) of the healthy-donor samples, IL-4 production decreased by 50%.

**EFFECT OF BEXAROTENE ON IFN-γ PRODUCTION**

It has previously been demonstrated that retinoic acid receptor (RAR)-specific retinoids, including ATRA and 13-cis retinoic acid, induce IFN-γ production and thus may mediate some of their beneficial therapeutic effects in CTCL through enhancement of IFN-γ-stimulated cell-mediated immunity. Thus, we determined whether bexarotene also exhibits this property. The PBMCs from 6 patients with medium tumor burden and 6 healthy vol-
unteers were cultured in media, 50 U of IL-2, 10µM bexarotene, 1-ng/mL ATRA, and 10µM bexarotene or 1-ng/mL ATRA in the presence of 50 U of IL-2. Among the PBMCs of patients, IFN-γ release increased an average of 3-fold (range, 0- to 9-fold) or 309% with the combination of ATRA and IL-2 vs IL-2 treatment alone. In all patient samples, treatment with ATRA in the presence of IL-2 demonstrated synergistic potentiation of IFN-γ production, as defined by a response that is greater than the additive sum of the response due to ATRA or IL-2 alone. Treatment with the combination of bexarotene and IL-2 was similar to treatment with bexarotene alone, with no enhancement of IFN-γ appreciated.

Among healthy volunteer samples, when treatment with 1-ng/mL ATRA and IL-2 was compared with treatment with IL-2 alone, an increase in IFN-γ production was appreciated in 5 of 6 samples. On average, IFN-γ release increased less than 1-fold, or 45%, for the combination of ATRA and IL-2 vs IL-2 treatment alone. Results from a representative patient with medium tumor burden and a healthy volunteer are depicted in Figure 5. As previously demonstrated, IL-2 treatment resulted in a much higher level of IFN-γ production among the PBMCs of healthy volunteers compared with those of the patient group.14 Thus, the absolute change in IFN-γ production in healthy volunteer samples treated with ATRA and IL-2 resulted in a smaller percentage of increase compared with treatment with IL-2 alone. Synergistic potentiation of IFN-γ release was observed in 4 of the 6 samples from healthy volunteers. Treatment with the combination of bexarotene and IL-2 was similar to treatment with bexarotene alone, and no enhancement of IFN-γ was observed.

The retinoid receptors (RAR and RXR) are members of a family of transcription factors belonging to the nuclear hormone receptor superfamily, which also includes peroxisomal proliferator activator receptor, thyroxine receptor, and vitamin D receptor.15 Retinoid X receptor is unique in that it may form a heterodimer with each member of the nuclear hormone receptor superfamily. The RAR-RXR heterodimer complex activates retinoid response elements that reside in gene promoter regions encoding for transcription factors, structural proteins, and cell surface receptors.15-17 Activation results in transcriptional modulation of cell function and differentiation, growth inhibition, and apoptosis.

Although much of the molecular basis of bexarotene’s mechanism of action remains unknown, previous studies have examined the effect of bexarotene on apoptosis, cell proliferation, differentiation, and tumorigenesis in immortalized cell lines. To our knowledge, this study is the first to evaluate T lymphocytes derived directly from SS patients with CTCL.

The results of this study support a mechanism of action of bexarotene that includes the induction of apoptosis of a portion of the malignant T-cell population. Apoptosis of malignant T cells from the peripheral blood of patients with CTCL and a high tumor burden was demonstrated with both 1µM and 10µM concentrations of bexarotene. This was observed after 72 hours of treatment and differs from a previous study that reported no growth inhibition at a concentration of 10µM for 24 to 72 hours in cell lines.3 The T lymphocytes from 67% of SS patients with high tumor burden repeatedly manifested high levels of apoptosis after culture with 10µM bexarotene for 72 hours, whereas T cells from 33% of patients consistently failed to demonstrate apoptosis on results of a repeat assay. This is similar to the clinical response rate in patients with advanced stages of CTCL receiving oral bexarotene. Dose dependency was evident with an average increase in apoptosis of 16.7% for the 1µM treatment group (n=3) and
49.5% for the 10μM treatment group (n = 6). Dose-dependent apoptosis in CTCL cell lines (MJD, HTU78, and HH), as evidenced by an increased number of cells with sub-G1 population and annexin V binding, was previously demonstrated by Zhang et al. Activation of caspase-3, an intracellular protease important in the degradation of intracellular substrates within the apoptotic pathway, with subsequent cleavage of poly-adenosine diphosphate-ribose polymerase, was also observed. The concentrations of bexarotene used in this study correlate with plasma levels documented in clinical trials.1,18,19

Previous treatments that induce apoptosis of T lymphocytes (eg, extracorporeal photopheresis) may have resulted in an increased sensitivity of patients’ cells to the effects of bexarotene. Therapies targeting different mechanisms of apoptosis may be additive or synergistic in the treatment of CTCL. This study evaluated combination treatment of patients’ cells with bexarotene and IFN-α, and no increase in apoptosis was demonstrated. It is likely that the improvement in clinical response of SS patients treated with bexarotene and IFN-α is due to a different mechanism of action, such as augmentation of cell-mediated cytotoxicity or an antiproliferative effect. Low-dose oral bexarotene in combination with IFN-α has been frequently used at our institution and appears to be associated with accelerated clearing of CTCL disease manifestations.9

No research to date has reported the effects of bexarotene on cytokine production. The in vitro effect of bexarotene on IL-4 production was examined in this study. In 45% of patients with high tumor burden undergoing evaluation, we found evidence of down-regulation of T_{H2} (IL-4) cytokine production from isolated PBMCs. Decreased IL-4 production was evident in 4 (67%) of 6 patients who demonstrated apoptosis after bexarotene treatment. No change or an increase in IL-4 production correlated with resistance to apoptosis in all patient samples.

Previous studies have determined that a T_{H2} cytokine pattern dominates in the peripheral blood and tissue of SS patients,10-13 and the malignant clone is of the TH2 phenotype.20,21 Increased levels of IL-4, a potent inducer of T_{H2} cell differentiation, was recently documented in the cytoplasm of T cells in advanced-stage CTCL and was speculated to play a significant role in disease progression.22 There is also evidence that levels of IL-4 in SS patients decrease and return to normal during remission and treatment with extracorporeal photopheresis.23-25 Similarly, the therapeutic effects of bexarotene may involve the down-regulation of IL-4 production, as was observed in nearly 50% of the malignant T cells from SS patients undergoing evaluation in this study.

Inhibition of nuclear factors of activated T cells may also result in reduced IL-4 production by controlling the transcription of T_{H2} cytokines that predominate in CTCL.10,11,12,13 Alternatively, the activated RAR–peroxisomal proliferator activator receptor heterodimer may compete with cytokine transfer factors nuclear factor of activated T cells (NFAT) and nuclear factor κB (NF-κB) for coactivators, resulting in decreased transcription of cytokines.15

The results of this study are consistent with previously published data that demonstrated that RAR-specific retinoids synergize with IL-2 to augment IFN-γ production by human PBMCs.14 Augmentation of IFN-γ production is of therapeutic benefit to patients with CTCL who exhibit depressed levels of type 1 helper T-cell (T_{H1}) cytokines (IL-2 and IFN-γ) and defects in cell-mediated immunity. Treatment with IFN-γ has been shown to reverse the cytokine abnormalities that occur in SS patients.26 Unlike the effects of RAR-specific retinoids, those of bexarotene do not involve the augmentation of IFN-γ. However, by decreasing levels of IL-4, a T_{H2} cytokine, bexarotene may aid in the induction of T_{H1} responses, including IFN-γ production.

CONCLUSIONS

Bexarotene treatment at clinically relevant concentrations induced apoptosis of T lymphocytes from patients with CTCL and SS that was not further enhanced by IFN-α. The frequency of in vitro apoptosis was similar to the clinical response rate observed in patients treated with bexarotene. Inhibition of in vitro IL-4 production from PBMCs with high tumor burden in response to mitogen stimulation correlated with susceptibility to apoptosis. This finding suggests that bexarotene may also act through down-regulation of T_{H2} cytokines, thereby enhancing T_{H1} responses.

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