Immunomodulatory effects of RXR rexinoids: modulation of high-affinity IL-2R expression enhances susceptibility to denileukin diftitox

Gullu Gorgun and Francine Foss

Rexinoids binding to both the retinoic acid receptor (RAR) and retinoid X receptor (RXR) families of rexinoid receptors have demonstrated clinical activity in hematologic malignancies and have been shown to mediate genes associated with both growth and differentiation. RXR rexinoids have demonstrated efficacy in the treatment of cutaneous T-cell lymphomas, but the mechanism of action is unclear. We explored the immunomodulatory effects of RAR and RXR rexinoids in human T- and B-cell leukemia cells and demonstrated that RXR rexinoids are capable of up-regulating high-affinity interleukin-2 receptor (IL-2R) expression. Exposure to $10^{-6}$ to $10^{-10}$ M bexarotene or Panretin for 48 hours was associated with increased expression of both the p55 and p75 subunits of the IL-2R in T-cell leukemia and p75 in B-cell leukemias. Furthermore, rexinoid exposure enhanced susceptibility of the cells to denileukin diftitox fusion toxin-targeting and -intoxicating cells expressing high-affinity IL-2R. These results suggest a rationale for combining rexinoids with IL-2R-targeted therapies in lymphoid malignancies as well as possible in autoimmune diseases. (Blood. 2002;100:1399-1403)

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Materials and methods

Cell culture
Cutaneous T-cell lymphoma (CTCL) NCI-HUT78, HUT102, pre-B cell line NALM6, and acute lymphoblastic leukemia cell line CEM were obtained from the American Type Culture Collection (Manassas, VA). Fresh cells from patients with CTCL and B-CLL were isolated by Ficoll-Hypaque centrifugation. Cells were grown in complete medium (RPMI-1640 medium including 10% fetal bovine serum, 50 μg/mL glutathione, 50 μg/mL streptomycin, and 50 μg/mL penicillin).

Chemicals
Denileukin diftitox (Ontak, DAB 389 IL2), alitretinoin (Panretin, LGD 1057; 9-cis retinoic acid; 3,7-dimethyl-9-[2,6,6-trimethyl-1-cyclohexen-1-yl]-2-trans-4-trans-6-cis-8-trans-nonatetraenoic acid), and bexarotene (Targretin, LGD 1069; 4-[1-(3,5,5,8,8,-pentamethyl-5,6,7,8-tetrahydro-2-naphthalenyl) ethenyl] benzoic acid) were obtained from Ligand Pharmaceuticals.
hours and then labeled with phycoerythrin (PE)–conjugated monoclonal antihuman CD25 PE (IL-2Rα) and CD122 PE (IL-2Rβ) (Becton Dickinson, CA) antibodies, fixed, and analyzed using FACScan (Becton Dickinson, Beckman Instruments, San Jose, CA) Laser excitation was set at 488 nm.

**IL-2R subunit analysis by Western immunoblot**

Cells (5 × 10^5 cells/mL) were incubated with 10^{-6} M and 10^{-8} M rexinoids for 48 hours and lysed; proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes at 100 V for 1 hour. Immunoblots were performed using antibodies to human IL-2Rα, IL-2Rβ, and IL-2Rγ (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were detected by Western blot chemiluminescence reagents (NEN Life Science, Boston, MA) and exposed on Kodak XAR film (Rochester, NY). The protein bands were stained by Coomassie blue to demonstrate the amount of protein loaded per lane. The constitutively expressed Coomassie blue–stained protein bands were used as control.

**Inhibition of protein synthesis by[^14]C-leucine incorporation assay**

Cells were seeded in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA), 5 × 10^3 cells/100 μL in complete medium, and treated with 10^{-6} to 10^{-10} M rexinoids for 48 hours. Cells were exposed to 10^{-6} to 10^{-14} M denileukin diftitox for an additional 72 hours. The plates were centrifuged for 5 minutes at 170g, and the medium removed and replaced with 100 μL leucine-free medium containing 0.25 μCi[^14]C-leucine (0.00025 MBq; New England Nuclear, Boston, MA). Cells were incubated at 37°C for 3 hours; then protein was precipitated by trichloroacetic acid (TCA) and harvested on glass fiber filters. Incorporation of[^14]C-leucine was quantified by scintillation counting. All assays were performed in triplicate and results were reported ± SEM of triplicate assays.

**MTT cell cytotoxicity assay**

Cells were seeded in triplicate in 96-well plates in 50 μL complete media at a density of 5 × 10^3 cells/well. The 50-μL aliquots of rexinoids were added to each well to give a range of concentrations from 10^{-6} to 10^{-10} M in a final volume of 100 μL. After 48 hours of incubation, 20 μL denileukin diftitox was added to each well to give various concentrations from 10^{-6} to 10^{-10} M. Cells were incubated for an additional 72 hours. Then 20 μL MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide salt) from 5 mg/mL stock was added at the last day and incubated for 4 hours. The formazan crystals were solubilized by adding 100 μL 20% SDS in 50% dimethyl formamide (DMF; pH 4.7) and incubating at 37°C overnight. The absorbance of the formazan product was measured on an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm.

**Results**

We and others demonstrated that the CTCL cell line HUT78, acute T-lymphoblastic leukemia cell line CEM, and the pre-B cell leukemia cell lines NALM6 and Raji express low-affinity IL-2R, consisting of the p55 (IL-2Rα) and p64 (IL-2Rγ) subunits and are thereby resistant to killing by denileukin diftitox (Ontak), whereas HUT102 T-leukemia cells expresses high-affinity IL-2R (p55, p75, p64) and are efficiently intoxicated by denileukin diftitox.11

**Effects of rexinoids on T- and B-leukemia cells**

To determine the effects of rexinoids on high-affinity IL-2R expression, we exposed T- and B-leukemia cell lines and fresh leukemia cells to...
bexarotene, an RXR ligand, and alitretinoin, which binds to both RXR and RAR receptors. After a 48-hour incubation, there was no inhibition of cell growth by MTT and, morphologically, no induction of apoptosis in the treated cells. Expression of IL-2R subunits after rexinoid exposure is shown in Figure 1.

Both alitretinoin and bexarotene up-regulated the cell surface expression of p55 and p75 at least 4-fold in HUT78 cells and fresh CD4+CD7+ Sezary cells, which lacked IL-2R expression by immunohistochemistry. Rexinoids had less effect on IL-2R expression in NALM6 and Raji cells and in human B-CLL cells, with increased the expression of p75 but not p55 (Figure 1C).

Immunoblots confirmed that both alitretinoin and bexarotene increased the expression of p55 and p75 in HUT78 (Figure 2A,B) and fresh Sezary cells and only p75 in the B cells (Figure 2C).

RXR-selective rexinoid bexarotene (Targretin) and RAR/RXR-specific rexinoid alitretinoin (Panretin) enhanced sensitivity of T and B cells to denileukin diftitox (Ontak)

To test modulation of expression of functional high-affinity IL-2R after rexinoid exposure, we expose rexinoid-treated cells to denileukin diftitox and measured protein synthesis inhibition and cell growth. Because the mechanism of cytotoxicity of denileukin diftitox is related to the diptheria toxin–mediated adenose diphosphate ribosylation of elongation factor 2, inhibition of protein synthesis is indicative of efficient receptor-mediated internalization of the fusion toxin. As shown in Figure 3, panels A and B, a 50% to 70% inhibition of protein synthesis was demonstrated in HUT78 and fresh Sezary cells after exposure to alitretinoin or bexarotene, respectively. In the B cells, alitretinoin inhibited the protein synthesis by 35% compared to over 50% with bexarotene (Figure 3C). As shown, low doses of denileukin diftitox alone were associated with increased proliferation, presumably due to cytokine-mediated signaling events from binding of denileukin diftitox to intermediate- and low-affinity receptor-expressing cells.12

To confirm that inhibition of protein synthesis corresponded to increased cytotoxicity, MTT assays were performed. Bexarotene had a greater effect on enhancing cytotoxicity of denileukin diftitox than alitretinoin in both T- and B-leukemia cells, concordant to the degree of protein synthesis inhibition (Figure 4).

The results of the cytotoxicity assays are summarized in Table 1, which demonstrates that bexarotene alone had no effect on cell number.

Discussion

Vitamin A and its analogues influence differentiation and proliferation and may also alter immune responses. Rexinoids exert their effects by binding to the rexinoid A receptor (RARαβγ) or the
rexinoid X receptor (RXRαβγ) in the nuclei of cells, either directly or indirectly via nuclear transcription factors. As a result, rexinoids regulate the expression of a wide range of target genes.13

The effects of RAR and RXR rexinoids on IL-2R expression were determined in T and B lymphocytes, which do not express high-affinity IL-2R.11,14 IL-2Rα and IL-2Rβ expression is increased after exposure of the T cells to alitretinoin or bexarotene for 48 hours, whereas only IL-2Rβ expression was increased in B-CLL cells. These results demonstrate lineage-specific effects of rexinoids and suggest that modulation of IL-2Rα or IL-2Rβ may be in part related to the state of differentiation of the cell.

A number of factors have been shown to modulate expression of IL-2R subunits. The p55 subunit is induced by T-cell activation, viral gene products, protein kinase A (PKA), IL-2, IL-1, and tumor necrosis factor α (TNF-α).15 Likewise, the p75 subunit, which is expressed on resting T lymphocytes, is up-regulated by activation, IL-2, and IL-4. The p64, or common γ subunit, is constitutively expressed but up-regulated by IL-2 and interferon γ.

Up-regulation of IL-2R subunits by rexinoids is most likely mediated by modulation of the binding of transcriptional elements. In the case of the RAR rexinoid, ATRA, direct binding to the PML transcription factor in acute promyelocytic leukemia cells has been shown to be the mechanism of action.16 RXR rexinoids have been shown to bind directly to RXR transcriptional elements either as homodimers or heterodimers with RAR. RXR elements have been identified in a number of genes, including the thyroid-stimulating hormone promoter and the peroxisome proliferator-activated receptor promoter.16

The differential effect of rexinoids on IL-2R expression in T and B cells may be related to interaction with other transcriptional regulators. Both RAR and RXR rexinoids inhibit activation and prevent apoptosis in B lymphocytes while inducing cell differentiation and proliferation in T lymphocytes.17 IL-2Rα is not expressed on resting lymphocytes but is potently induced after activation.18 IL-2Rα is mainly regulated at the level of transcription by induction of nuclear factor-kB (NF-kB) and serum response element.19 Rexinoids may induce IL-2Rα expression by enhancing expression of IL-2Rβ in T lymphocytes.15

IL-2Rβ is linked to at least 2 intracellular signaling pathways mediating nuclear proto-oncogene induction. One pathway involves tyrosine phosphorylation by janus kinase 1 (JAK1) and src-family phosphotyrosine kinases (PTKs) and leads to the induction of c-fos and c-jun through the activation of p21.20 Another pathway involves induction of c-myc and bcl-2 genes.20 Up-regulation of IL-2Rβ, therefore, favors proliferation. Because IL-2Rγ is constitutively expressed in lymphocytes and, via janus kinase signal transducer and activation of transcription (JAK-STAT) phosphorylation, serves as a messenger to rapidly regulate expression of target genes,21 up-regulation of IL-2Rβ by the rexinoids enhances activation and proliferation signals even in the absence of up-regulation of IL-2-Rα in the B cells. This may explain the effects of the RXR rexinoids alone to induce the proliferation we observed in both T- and B-leukemia cells.

Up-regulation of functional high-affinity receptor by rexinoids could potentially confer susceptibility of the cells to IL-2R–targeted therapies. We demonstrate that rexinoid treatment enhanced the cytotoxicity of the IL-2R–directed fusion toxin, denileukin diftitox, in resistant T- and B-cell leukemia cells. The inhibition

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<th>Cell lines and cells</th>
<th>Expression of IL-2 receptor subunits (baseline)</th>
<th>Cytotoxicity (% survival) bexarotene</th>
<th>Cytotoxicity (% survival) bexarotene + Ontak (10⁻¹² M)</th>
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All the data represent cell count ± SEM by MTT assay.
of protein synthesis by denileukin diftitox requires receptor-mediated internalization of the fusion toxin and intracellular processing to cleave the enzymatically active moiety of diphtheria toxin into the cytosol.\textsuperscript{22-23} The susceptibility of both normal and neoplastic cells to denileukin diftitox--induced cytotoxicity is dependent on the expression of the high-affinity IL-2R, consisting of the p55 (α), p75 (β), and p64 (γ) subunits.\textsuperscript{24-26} The inhibitory concentration of 50% (IC\textsubscript{50}) of high-affinity IL-2R--expressing cells is 10 to 12 M, whereas cells expressing intermediate-affinity receptors (p75, p64) are intoxicated at an IC\textsubscript{50} of 10 to 10\textsuperscript{-1} M. Expression of high-affinity IL-2R is restricted to activated T lymphocytes, activated B lymphocytes, and activated macrophages, whereas intermediate-affinity receptors are found on natural killer cells and resting T cells.\textsuperscript{25-29}

In addition to up-regulation of high-affinity receptor, receptor exposure might have other effects on multiple signaling pathways to enhance apoptosis in the presence of the fusion toxin. Denileukin diftitox has been shown to initiate signal transduction with ligand engagement may contribute to the markedly enhanced cytotoxicity observed in this study.

Cell viability studies show that neither alitretinoin or bexarotene induces cell death at concentrations ranging from 10\textsuperscript{-6} to 10\textsuperscript{-8} M. Other studies have demonstrated that RXR rexinoids induce apoptosis in epithelial cell lines and in HL60 cells. RXRα has been shown to be down-regulated during the transition from G\textsubscript{0}/G\textsubscript{1} to S phase of the cell cycle, whereas RAR-γ has been shown to facilitate apoptosis of T lymphocytes, suggesting a role for rexinoid homodimers and heterodimers in cell cycle control in lymphocytes.\textsuperscript{7,30,31}

Our results demonstrate that RXR rexinoids up-regulate high-affinity IL-2R expression on human B- and T-cell leukemia cells. These results suggest that the clinical efficacy of denileukin diftitox might be enhanced by the addition of an RXR rexinoid, and phase I/II trials exploring this concept are under way. Further, bexarotene is widely used as a therapy for patients with CTCL, but its mechanism of action in this disease is unclear, because our studies demonstrate no direct cytotoxic effects on Sezary leukemia cells. The demonstration of an immunomodulatory effect of bexarotene to up-regulate high-affinity IL-2R may have consequences in the context of the generation of antitumor immunity in patients, and, likewise, it is unclear whether up-regulation of IL-2R might render the tumor cells more susceptible to fas-mediated cytotoxicity in vivo. Our results suggest that further studies of the in vivo immunomodulatory effects of bexarotene are warranted.

References

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