Role of Intracellular Redox Status in Apoptosis Induction of Human T-Cell Leukemia Virus Type I-infected Lymphocytes by 13-cis-Retinoic Acid

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ABSTRACT

We have shown that cell cycle progression of human T-cell leukemia virus type I (HTLV-I)-transformed T-cell lines was inhibited by 13-cis-retinoic acid (13cRA). In the present study, we report that 13cRA inhibited proliferation and induced cell death of peripheral blood mononuclear cells obtained from four patients with acute adult T-cell leukemia but not of mitogen- or interleukin 2-activated peripheral blood mononuclear cells from HTLV-I-negative healthy donors. Because HTLV-I-infected lymphocytes are susceptible to oxidative stress, we examined the role of the intracellular redox state in 13cRA-induced cell death using a HTLV-I-positive T-cell line, ATL2, as a model. 13cRA induced apoptosis in ATL2 cells within 48 h in a dose-dependent manner. The ability of 13cRA to induce apoptosis was more potent than that of all-trans-retinoic acid. Apoptosis induction by 13cRA was significantly enhanced by buthionine sulfoximine (BSO), which decreased the levels of intracellular reduced glutathione, although 13cRA by itself did not alter them, suggesting that intracellular reduced glutathione may modulate 13cRA-induced apoptosis. In addition, flow cytometric analysis revealed that 13cRA increased intracellular peroxides in 24 h and that the addition of BSO further enhanced them. Although N-acetylcysteine had only a marginal effect, pretreatment with catalase markedly inhibited 13cRA-induced apoptosis. These results suggest that peroxide generation, i.e., oxidative stress, may play a crucial role in the induction of apoptosis by 13cRA and further demonstrate that combined treatment with 13cRA and BSO induces apoptosis of HTLV-I-positive lymphocytes even more potently.

INTRODUCTION

ATL, an aggressive and often fatal lymphoid malignancy, is etiologically associated with HTLV-I (1). However, the precise mechanism by which HTLV-I infection develops into leukemia remains poorly understood, and ATL remains resistant to most conventional chemotherapies. We have previously shown that 13cRA preferentially inhibits the proliferation of HTLV-I-transformed T-cell lines (2). RA derivatives of vitamin A and are known to exert a wide variety of effects on vertebrate development, cellular differentiation, and proliferation (3–5). Whereas diverse effects of atRA or 9cRA are known to participate in apoptosis induction in several virus infections including HIV type I (18, 19) and HTLV-I (1, 20). Recently, treatment of embryonic stem cells with atRA has been reported to increase intracellular ROIs (21). Furthermore, HTLV-I-positive cells are even more susceptible to oxidative stress (20, 22). This evidence prompted us to test the hypothesis that oxidative stress may be involved in 13cRA-driven growth inhibition and/or cell death of HTLV-I-positive lymphocytes. Here we report that 13cRA is a potent apoptosis inducer for HTLV-I-positive cells and have investigated whether intracellular redox status may play a role in 13cRA-induced apoptosis.

MATERIALS AND METHODS

Cells. PBMCs were isolated from peripheral venous blood of four patients with acute ATL as well as HTLV-I-negative healthy volunteers by Ficoll-Hypaque density gradient centrifugation. PBMCs, ATL2, a HTLV-I-positive T-cell line that was kindly provided by M. Maeda (Kyoto University, Kyoto, Japan), and Jurkat, a HTLV-I-negative T-cell line, were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated FCS (Filtron, Brooklin, Australia), 100 units/ml penicillin G, and 0.1 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2.

Reagents. 13cRA and atRA, purchased from Sigma Chemical Co. (St. Louis, MO), were dissolved at 10–2 M in 100% DMSO. The final DMSO concentrations in the medium were no higher than 0.1% in which control experiments showed no effect on the proliferation or viability of the cells used (data not shown). BSO, NAC, and catalase (bovine liver, thymol-free; 0.048 μg/unit) were also purchased from Sigma Chemical Co. and dissolved in PBS.

Proliferation Assay and Evaluation of the Percentage of Viable Cells. The proliferation of PBMCs was evaluated by [3H]thymidine incorporation as described. Briefly, 100 μl of 105 cells/ml were cultured in 96-well microplates (Nunc, Naperville, IL) in triplicate samples for the number of indicated hours, pulsed with [3H]thymidine at 1 μCi/well for 4 h, and harvested. Incorporated [3H]thymidine was counted in a liquid scintillation counter (Alkota, Tokyo, Japan). Viable cell numbers and the percentage of viable cells were assessed by trypan blue dye exclusion test using a hemacytometer.

Quantitation of the Percentage of Apoptotic Cells. The percentage of apoptotic cells was assessed by fluorescence microscopy, after staining with AO and EB, as described previously (23). Briefly, 1 μl of stock solution containing 100 μg/ml AO and 100 μg/ml EB was added to 25 μl of cell suspension. After mixing, the cells were examined by fluorescence microscopy, after staining with AO and EB, as described previously (23). Briefly, 1 μl of stock solution containing 100 μg/ml AO and 100 μg/ml EB was added to 25 μl of cell suspension. The characteristic appearance of apoptosis, including cell shrinkage, blebbing, and apoptotic bodies, were counted manually.
Fig. 1. The effect of 13cRA on proliferation and cell viability of PBMCs from four different patients with ATL (a) and PHA- or IL-2-activated PBMCs from a HTLV-I-negative healthy donor (b). PBMCs (10^6 cells/ml) from patients with ATL were cultured with or without the indicated concentrations of 13cRA for 4 days and then harvested for [3H]thymidine incorporation and trypan blue dye exclusion. PBMCs (10^6 cells/ml) from healthy donors were cultured with or without the indicated concentrations of 13cRA in the presence of 0.1% PHA or 100 IU/ml rIL-2 for 3 days and then harvested for [3H]thymidine incorporation and trypan blue dye exclusion. A representative of four different healthy donors that were all comparable is shown. Columns, [3H]thymidine incorporation; ○, percentage of viable cells.

The proportion of cells undergoing apoptosis was also measured by flow cytometric analysis after staining with PI (Sigma) as described previously (24). In brief, cell pellets were suspended in 1 ml of hypotonic fluorochrome solution containing 50 μg/ml PI in 3.4 mM sodium citrate, 0.1% Triton X-100, 1 mM Tris (pH 8), and 0.1 mM EDTA and incubated in the dark at 4°C overnight. The PI fluorescence of individual nuclei was measured, and the percentage of cells with hypodiploid DNA was calculated with a flow cytometer (Ortho, Raritan, NJ).

**Immunoblot Analysis.** Immunoblot analysis was performed as described previously (25). Briefly, 10^6 cells were lysed on ice in 0.5% NP40 lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 50 mM p-nitrophenyl-p'-guanidinobenzo-
OXIDATIVE STRESS IN RA-INDUCED APOPTOSIS

Measurement of Intracellular GSH. Intracellular GSH contents were measured using the Glutathione Assay kit (Calbiochem, San Diego, CA). In brief, 2 × 10⁶ cells were treated with the indicated doses of RAs, BSO, and/or NAC for 48 h and homogenized in 5% metaphosphoric acid using a Teflon pestle. Cell lysates were assayed according to the manufacturer’s instructions.

Detection of Peroxides Accumulated in ATL2 Cells by Flow Cytometry. ATL2 cells (2 × 10⁶ cells/ml) were cultured with or without indicated treatments for 24 h and washed with PBS. Then cells were recultured in fresh RPMI 1640 with 10% FCS and 5 μM DCFH-diacetate (Molecular Probes, Inc., Eugene, OR) for 30 min at 37°C, and the fluorescence intensity was measured by flow cytometer.

RESULTS

13cRA Inhibited Proliferation and Induced Cell Death of PBMCs from Patients with Acute ATL but not PBMCs from Normal Donors. We have shown that 13cRA inhibited proliferation of HTLV-I-positive but not HTLV-I-negative T-cell lines (2). To determine whether 13cRA-induced growth inhibition is generalizable to other HTLV-I-infected lymphocytes, we investigated the effects of 13cRA on cell growth and viability of PBMCs obtained from four patients with acute ATL. Although there was individual variability...
among the patients, 13cRA inhibited the proliferation and reduced the percentage of viable cells of PBMCs in a dose-dependent manner in all four cases (Fig. 1a). In contrast, 13cRA did not alter the viability of unstimulated PBMCs obtained from HTLV-I-negative healthy donors (data not shown). In addition, to evaluate whether 13cRA affects the physiological response of PBMCs to mitogens or IL-2, we further tested the effects of 13cRA on proliferation and viability of PHA- or rIL-2-activated PBMCs from HTLV-I-negative donors. As shown in Fig. 1b, 13cRA did not significantly affect their proliferation or viability, suggesting that 13cRA can selectively induce apoptosis of HTLV-I-infected cells without suppression of normal immune response.

13cRA Induced Apoptosis of ATL2 Cells More Potently than Did atRA. To elucidate the mechanism by which 13cRA induces the death of HTLV-I-positive lymphocytes, we used ATL2 cells as a model. We first confirmed that 13cRA inhibited the proliferation of ATL2 cells but not of HTLV-I-negative Jurkat cells (Fig. 2, a and b). Next we showed that 13cRA induced apoptosis of ATL2 cells in 48 h in a dose-dependent manner (Fig. 3, a and b). As shown in Fig. 3a, 13cRA had more potent activity in apoptosis induction than did atRA, which is known to exert a variety of biological effects through binding to nuclear receptor RAR. Because the binding affinity of 13cRA to RAR is much lower than that of atRA (11), an explanation of this result may be related to their solubility, cell uptake of ligands, metabolism of ligands, RAR isoform selectivity of 13cRA versus atRA, or a pathway distinct from RAR.

RAs Did Not Affect the Expression of Bcl-2, Bax, or Fas in ATL2 Cells. A number of pathways leading to apoptosis are regulated by the expression of Bcl-2 as well as Bax (27, 28). Therefore, we examined the effect of 13cRA or atRA on the expression of Bcl-2 or Bax in ATL2 cells by immunoblot analysis. As shown in Fig. 4a, neither RA affected the expression of either Bcl-2 or Bax at 10 μM, at which they induced apoptosis of ATL2 cells. We also investigated the expression of Fas on the surface of ATL2 cells treated with RAs by flow cytometry, because Fas plays a major role in the induction of apoptosis, particularly in activated lymphocytes (29). However, RAs did not show any effects on their Fas expression (Fig. 4b).

BSO Enhanced 13cRA-induced Apoptosis of ATL2 Cells. There is growing evidence that the intracellular redox status plays an important role in the regulation of cell death (15, 30, 31). GSH is one of the major components controlling intracellular redox status (32). Therefore, we next examined the effects of NAC and BSO, which are known to increase and decrease intracellular GSH contents, respectively (33, 34), on 13cRA-induced apoptosis. Fluorescence microscopy (Fig. 5a) as well as flow cytometry (Fig. 5b) revealed that a combination of 13cRA with 500 μM BSO resulted in a higher percentage of apoptosis than did 13cRA alone. Furthermore, whereas DNA ladder formation was not detected after a single treatment with 10−7 M 13cRA, it was clearly detectable in cells treated with the same amount of 13cRA in the presence of 500 μM BSO (Fig. 5c). BSO alone had no ability or only a weak ability to induce apoptosis (Fig. 5, a–c). Furthermore, in the presence of 10 mM of NAC, the percentage of apoptosis of 13cRA-treated ATL2 cells was decreased by no more than about 20% (Fig. 5a). These findings suggest that intracellular GSH may modulate apoptosis induction by 13cRA, although GSH imbalance alone does not lead to apoptosis. Augmentation of apoptosis by BSO was also seen in the case of atRA, although the percentage of apoptosis was lower than that of 13cRA (data not shown), suggesting that apoptosis induction by atRA may involve a mechanism similar to 13cRA.

Intracellular GSH Content Was Not Altered in 13cRA-treated ATL2 Cells but Was Decreased by BSO. To confirm whether intracellular GSH is involved in 13cRA-induced apoptosis, we meas-
Table 1 Measurement of intracellular GSH contents in treated ATL2 cells

ATL2 cells were cultured with the indicated concentrations of 13cRA in the presence or absence of 500 μM BSO or 10 mM NAC for 48 h. Intracellular GSH contents (nmol/10^6 cells) were measured as described in "Materials and Methods." The means ± SD of three independent experiments are shown.

<table>
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<tr>
<th>13cRA(μM)</th>
<th>Control*</th>
<th>500 μM BSO</th>
<th>10 mM NAC</th>
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<tr>
<td>00</td>
<td>4.37 ± 0.45</td>
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<tr>
<td>10</td>
<td>3.91 ± 0.45</td>
<td>2.00 ± 0.23</td>
<td>3.93 ± 0.41</td>
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*ATL2 cells were treated with the indicated concentrations of 13cRA alone.

Fig. 5. a, the effects of BSO or NAC on the percentage of apoptosis of 13cRA-treated ATL2 cells. ATL2 cells (2 × 10^6 cells/ml) were cultured in the presence or absence of 500 μM BSO or 10 mM NAC and harvested for AO and EB staining after 48 h of culture. The means and SD (vertical bars) among three different experiments are shown. b, flow cytometric analysis of 13cRA-induced apoptosis of ATL2 cells. ATL2 cells were cultured with or without 10^-7 M 13cRA, 500 μM BSO, and 10^-7 M 13cRA + 500 μM BSO for 48 h and analyzed for the percentage of hypodiploid DNA by flow cytometry after PI staining. c, DNA ladder formation in 13cRA- and/or BSO-treated ATL2 cells. DNA was isolated from ATL2 cells with indicated treatments for 48 h and electrophoresed as described in "Materials and Methods." Lane 1, molecular size marker; Lane 2, no treatment; Lane 3, 500 μM BSO; Lane 4, 10^-8 M 13cRA; Lane 5, 10^-7 M 13cRA; Lane 6, 10^-6 M 13cRA; Lane 7, 10^-7 M 13cRA + 500 μM BSO.

ured intracellular GSH contents in untreated or treated ATL2 cells (Table 1). Whereas 10^-8^-10^-5 M 13cRA had no effects on intracellular GSH content, 500 μM BSO alone or together with 13cRA decreased it by approximately 50%, suggesting that BSO may facilitate 13cRA-induced apoptosis through the reduction of intracellular GSH level. Next we examined the effect of NAC on intracellular GSH content. Even at 10 mM, NAC failed to alter GSH levels in ATL2 cells. A limited inhibitory effect of NAC against 13cRA-induced apoptosis (no more than 20% inhibition) may be explained by a reducing activity of NAC itself rather than an increase in GSH. These results suggest that whereas 13cRA alone does not significantly affect intracellular GSH contents, BSO may increase 13cRA-induced apoptosis by decreasing the intracellular GSH levels.

13cRA Alone Increased Intracellular Peroxide Accumulation, and BSO Enhanced It Further. We next assayed intracellular peroxides to assess whether 13cRA, in the absence or presence of BSO, modulates intracellular redox status in ATL2 cells through attenuation of peroxide levels. We first confirmed that the interference of DCFH fluorescence by RAs was not observed after pretreatment of ATL2 cells with 1 and 10 μM RAs for only 1 h and that a micromolar concentration of retinol, which is another lipid-like agent similar to
added to the culture medium 1 h before treatment with 13cRA. As shown in Fig. 7, pretreatment with catalase inhibited apoptosis induced by 13cRA in a dose-dependent manner (about 50% inhibition by 10,000 units/ml catalase). This finding suggests that intracellular accumulation of peroxides may be involved in cell death elicited by 13cRA.

**DISCUSSION**

This study demonstrated the ability of 13cRA to induce cell death of HTLV-I-positive cells, including PBMCs from patients with ATL as well as the ATL2 cell line. In addition, 13cRA induced apoptosis of ATL2 cells more potently than did atRA, despite a weak binding affinity of 13cRA for the nuclear receptors (RAR or RXR). Most studies of apoptosis induction by retinoids, such as atRA, 9cRA, or a synthetic RA, 4-hydroxyphenyl retinamide, have revealed that RAR and/or RXR are involved in the signaling pathway leading to apoptosis (35—39). Furthermore, recently, the activation of RAR and/or RXR has been shown to decrease the expression of Bcl-2 (40—42). Okazawa et al. (43) reported that overexpression of Bcl-2 inhibited atRA-induced apoptosis of embryonal stem cells. Thus, the downmodulation of Bcl-2 expression is believed to be one of the mechanisms of RA-induced apoptosis. However, as for 13cRA, there has never been evidence regarding the involvement of either the nuclear receptors or Bcl-2 expression in the induction of apoptosis. According to our experiments, at least protein levels of bcl-2 were not altered by treatment with 13cRA or atRA, although it was still unclear whether bcl-2 functions remained the same after the treatment. We have also confirmed no effects of either 13cRA or atRA on the expression of other apoptosis-related proteins, Bax and Fas. Because HTLV-I-infected cells are known to be more susceptible to oxidative stress than HTLV-I negative cells (1, 22), we explored the role of intracellular redox status in the induction of apoptosis by 13cRA.

Here we showed that BSO enhanced 13cRA-elicited apoptosis of ATL2 cells through the reduction of intracellular GSH levels, whereas 13cRA alone did not affect GSH contents. These results suggest that intracellular GSH levels may modulate the sensitivity of ATL2 to 13cRA. GSH, a free radical scavenger, is required for protecting cells from oxidative stress (32). BSO has been reported to enhance the anticancer effects of several drugs such as Adriamycin, melphalan, RAs, did not show any effects on apoptosis induction or peroxide generation (data not shown). As shown in Fig. 6, 13cRA as well as BSO increased intracellular peroxide accumulation in the 24 h after the treatment. The ability of 13cRA to increase peroxide contents was more potent than that of atRA. BSO also had an additive effect on the accumulation of peroxides in 13cRA-treated cells. These results indicate that the ability of 13cRA or atRA to induce apoptosis of ATL2 cells correlated with the increase of intracellular peroxide contents. Therefore, we hypothesized that 13cRA-induced production of peroxides might result in apoptosis induction in ATL2 cells.

**Pretreatment with Catalase Protected 13cRA-treated ATL2 Cells from Apoptosis.** To confirm the hypothesis, we examined whether catalase, which quenches hydrogen peroxide, blocks 13cRA-induced apoptosis of ATL2 cells. Catalase (500—10,000 units/ml) was added to the culture medium 1 h before treatment with 13cRA.
and cisplatin, most of which are known to induce apoptosis by the production of ROIs (44–46). Therefore, to elucidate whether ROI production is involved in 13cRA-induced apoptosis, we measured intracellular peroxide levels in 13cRA-treated ATL2 cells. Interestingly, flow cytometric analysis revealed that 13cRA treatment induced the accumulation of intracellular peroxide products in 24 h more potently than did aTRA and that BSO enhanced them further. The increase in peroxides preceded the induction of apoptosis that was detected 48 h after treatment. In addition, pretreatment with catalase, which has been reported to affect the intracellular redox status (16, 47), partially protected 13cRA-treated ATL2 cells from apoptosis. These results suggest that generation of peroxides or oxidative stress is an important biological step in 13cRA-induced apoptosis of HTLV-I-positive lymphocytes. Furthermore, our data are consistent with other reports regarding the relationship of oxidative stress to RA-induced apoptosis. Delia et al. showed that horseradish peroxidase-induced apoptosis was inhibited by some antioxidants, including NAC, ascorbic acid, α-tocopherol, and deferoxamine (40). The apoptosis of embryonic stem cells by aTRA was reported to be blocked by catalase, superoxide dismutase, or phenol (21). Therefore, ROI generation, i.e., oxidative stress, may generally occur by treatment with RAs.

We have reported that 13cRA potently inhibits the TRX/TRX reductase (Trx) system (2, 48), which protects cells against oxidative stress through scavenging ROIs (12, 13, 49, 50), in cell-free studies. Therefore, it is possible that suppression of the activity of TRX/Trx may participate in the increase in peroxides by 13cRA. By contrast, we found that 13cRA did not decrease the intracellular GSH levels, despite a rise in intracellular peroxides, suggesting that the GSH/GSH reductase system may not be affected in the presence of 13cRA. Therefore, the results that BSO enhanced both apoptotic induction and peroxide generation by 13cRA can be explained by dysregulation of these two critical redox systems. However, 13cRA may encourage peroxide production via another mechanism like respiratory burst, because 13cRA and aTRA were reported to increase phagocytosis and production of ROIs in human endothelial cells (51). We need more investigations as to how oxidative stress is generated after RA treatment.

In conclusion, we propose that 13cRA leads to apoptosis of HTLV-I-infected lymphocytes through the generation of peroxides, i.e., oxidative stress. It is suggested that the intracellular GSH contents may modulate 13cRA-induced apoptosis. Combined treatment with 13cRA and BSO is a potent inducer of apoptosis in HTLV-I-infected lymphocytes and may be a potential therapy for HTLV-I-related disorders.

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Oxidative stress in RA-induced apoptosis.


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