ANTICANCER RESEARCH *25*: 1945-1952 (2005)

Intracellular Glutathione Levels Determine Cell Sensitivity to Apoptosis Induced by the Antineoplasic Agent N-(4-hydroxyphenyl)retinamide

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Abstract. *Background: We have previously demonstrated that the synthetic retinoid N-(4-hydroxyphenyl) retinamide (4-HPR) induces the overproduction of reactive oxygen species (ROS) in human leukemia cells, which in turn triggers the intrinsic (mitochondrial) apoptotic pathway. In order to study the role of glutathione in 4-HPR-induced apoptosis, the levels of this antioxidant were analyzed in cell lines which are sensitive and resistant to the effects of 4-HPR, and the effect of the modulation of glutathione levels on 4-HPR cytotoxicity was characterized. Materials and Methods: Mitochondrial membrane potential ($\Delta\Psi m$) and the levels of glutathione were measured by flow cytometry. A fluorometric assay was used to measure intracellular ROS generation and Western blot was employed to analyze tissue transglutaminase expression. Results: 4-HPR generated large quantities of ROS in cell lines which expressed low glutathione levels, these cells being the most sensitive to the retinoid. The sensitivity of leukemia cells to 4-HPR could be modulated, either by increasing intracellular glutathione contents using all-trans retinoic acid (ATRA), or by decreasing it using DL-buthionine-S,R-sulfoximine (BSO). ATRA increased the level of expression of tissue transglutaminase, whereas inhibition of this enzyme led to enhanced apoptosis. Conclusion: Our findings indicate that the glutathione content contributes to determining the sensitivity of cells to 4-HPR and points to the potential application of glutathione-inhibiting agents as enhancers in 4-HPR-based therapies.*

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Key Words: N-(4-hydroxyphenyl) retinamide, apoptosis, reactive oxygen species, glutathione, tissue transglutaminase, BSO, all-trans retinoic acid (ATRA).

N-(4-hydroxyphenyl) retinamide (4-HPR), also called fenretinide, has potent chemopreventive effects in several animal models. It has emerged as one of the most promising alternatives to the natural, more toxic retinoids. Its anticancer activity seems to be related to its ability to inhibit tumor cell growth and to induce apoptosis (1-6). Many chemotherapeutic agents can trigger the mitochondrial apoptotic pathway by means of various stress signals (7). These signals can indirectly induce mitochondrial membrane permeabilization (MMP) or can have a direct action on mitochondrial proteins and lipids. In this sense, we have previously reported that 4-HPR induces apoptosis via reactive oxygen species (ROS) overproduction, which induces the loss of the mitochondrial transmembrane potential, in turn leading to the release of cytochrome *c* and caspase activation (8, 9).

Oxidative stress results in damage to most of the principal biological macromolecules and in potential apoptotic cell death (10). Antioxidants have been widely reported to protect against oxidative-stress-induced apoptosis. Glutathione (GSH) also plays a central role in intracellular antioxidant defense against free radicals, being the only built-in defense within mitochondria against excessive ROS production (11). This suggests that the cellular redox state may regulate cell survival and, thus, high GSH levels may provide resistance to oxidative stress-induced apoptosis (12-15).

Based on the evidence that the cellular redox state has an influence on the survival of malignant cells, it may also play a role in the response of these cells to agents such as 4-HPR. Consequently, agents which alter the redox state could modify the response of cells to 4-HPR. In the present work, the role of GSH in 4-HPR-induced apoptosis was studied by analyzing GSH levels in different cell lines and the effect of its modulation on cell survival.

Materials and Methods

Cell lines and culture conditions. CCRF-CEM and Jurkat, human acute lymphoblastoid leukemia cells, were grown in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml gentamycin and 2mM L-glutamine. A375 human melanoma cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 100U/ml penicillin/streptomycin. All culture cells were incubated at 37˚C under 5% CO_2.

Cell survival assay. A standard XTT assay (Roche Molecular Biochemicals, IN, USA) was used to determine cell survival. Cells were seeded in 96-well plates at a density of $1x10^6$ cells/ml for leukemia cells and $1x10^5$ cells/ml for melanoma cells (4 wells per experimental condition) and exposed to a range of 4-HPR concentrations. When indicated, CCRF-CEM cells were preincubated with all-trans-retinoic acid (ATRA, Sigma) 1nM 48 h and/or DL-buthionine-S,R-sulfoximine (BSO, Sigma), the γ-glutamylcysteine synthetase inhibitor, 50 μg/ml overnight. Eventually, CCRF-CEM and Jurkat cells were treated with the tissue transglutaminase (tTG) inhibitor (binding of an active site) monodansylcadaverine (MDC, Sigma) 70 μM overnight before 4-HPR. Survival was measured by the colorimetric assay. Absorbance was determined at 480 nm for each well. Cell survival percentages were calculated in each experiment in relation to the non-4-HPR- treated control cells.

Measurement of intracellular generation of ROS. We used the oxidation-sensitive fluorescent dye 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR, USA) to measure the production of ROS, as described previously (8). Briefly, cells seeded in 96-well culture plates were exposed to increasing 4-HPR concentrations. Eventually CCRF-CEM cells were preincubated with 1nM ATRA 48 h and/or 50 μg/ml BSO 16 h before 4-HPR treatment. After 1 h, cells were washed and incubated at 37˚C with 20 μg/ml DCFH-DA for 20 min. Fluorescence intensity was measured in a Fluoroskan Ascent (Labsystems). Four wells were used for each treatment.

Determination of intracellular GSH levels. Treated cells were incubated for 30 min at 37˚C in the dark with medium containing the GSH-reactive dye 5-chloromethylfluorecein diacetate (CMFDA, Molecular Probes) 10 μM (16). Cells were then resuspended in fresh medium and maintained in the same conditions for 30 min. The cells were washed and the emission of fluorescence was measured (at least 10,000 viable cells per sample) with a Coulter EPICS ELITE ESP flow cytometer (EPICS division, Coulter Corp.).

Analysis of mitochondrial membrane potential (ΔΨm). Variations of the ΔΨm after 1 nM ATRA and/or 3 μM 4-HPR exposure were evaluated by employing 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$, Molecular Probes). Cells were incubated with 100 nM $DiOC_6(3)$ in PBS for 20 min at 37˚C in the dark. After washing, 5 μg/ml IP per sample were added and viable cells were analyzed in Coulter EPICS ELITE ESP flow cytometer.

Western blotting. Cell pellets were resuspended in lysis buffer (SDS 1%, sodium orthovanadate 1 mM and Tris-HCl 10 mM, pH 7.4) and protease inhibitor cocktail (Roche) at $2x10^6$ cells per 100 μl. The viscosity of the lysates was reduced; first boiling for 5 min, and second passing three times through a 26 needle to shear the DNA. After centrifugation at 10,000 xg for 15 min, 45 μg of each protein supernatant were loaded on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose membrane and subjected to immunodetection of tTG and α-actin. Monoclonal antibody anti-tTG was purchased from NeoMarkers and anti-α-actin from Sigma; both were used 1:100 diluted.

Statistics. The results were expressed as the mean ± SD of at least three independent experiments. Student's two-tailed, unpaired *t*-test was used, and values of $p<0.05$ were considered to be significant.

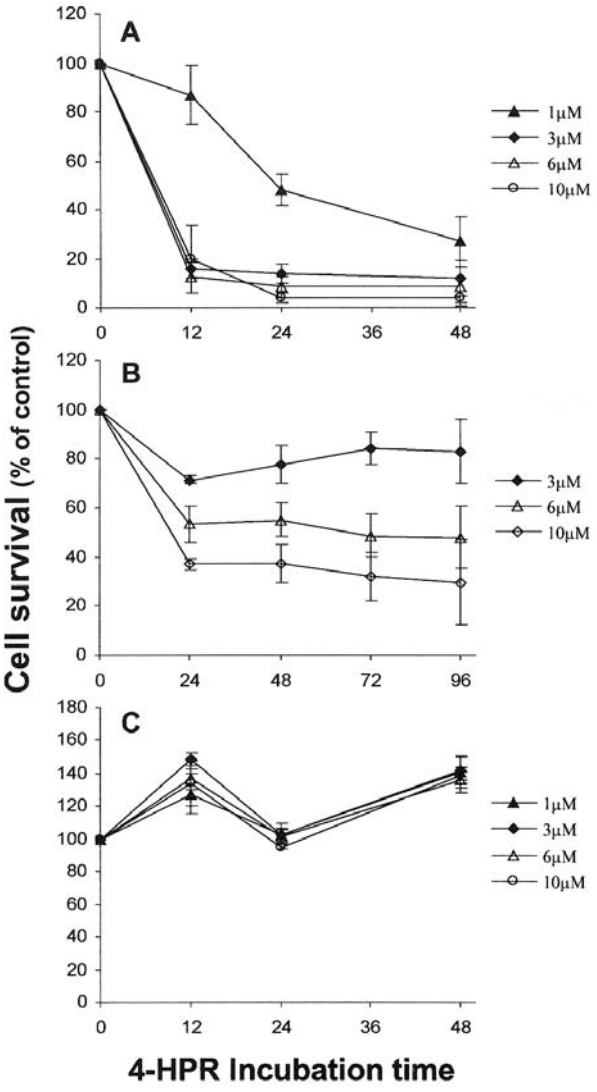

Figure 1. *Dose- and time-dependent effects of 4-HPR on the survival of (A) CCRF-CEM, (B) Jurkat and (C) A375 cell lines. The A375 cell line is most resistant to 4-HPR, whereas the CCRF-CEM cell line is most sensitive. Values represent the mean ± SD of three independent experiments (n ≥12).*

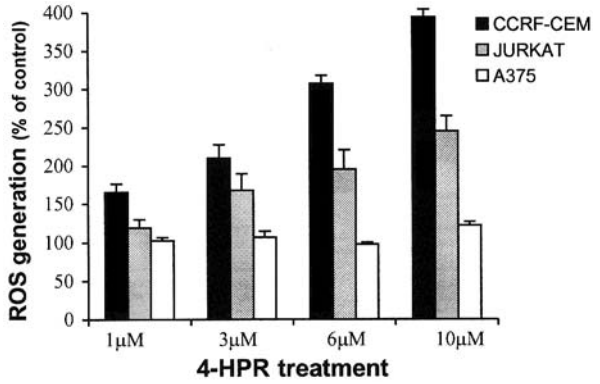

Figure 2. *Dose-dependent comparative study of ROS generation in 4-HPR-treated CCRF-CEM, Jurkat and A375 cells. The different cell lines were treated with increasing doses of 4-HPR for 1 h and ROS production was measured by plate fluorometry. Values represent the mean ± SD of three independent experiments. ROS generation was calculated as a percentage of that measured in control, non-4-HPR-treated cells.*

Results

4-HPR toxicity in different cell lines. We have previously demonstrated that 4-HPR induces a strong apoptotic response in CCRF-CEM leukemia cells (8, 9). In the present study, the dose and time dependence of the effect of 4-HPR on the survival of CCRF-CEM cells was characterized. Following 24-h treatment, cell death was observed in almost all cells (Figure 1A). In addition, 4-HPR induced cell death in Jurkat cells (Figure 1B). However, the extent of death was lower and the kinetics were slower than those observed in CCRF-CEM cells. Cell survival decreased by only about 20% after 96 h of 3 μM 4-HPR treatment and 10 μM 4-HPR was necessary to produce apoptosis in 72% of cells. Finally, neither 4-HPR-induced cell death nor cell growth inhibition were observed in A375 melanoma cells, which were resistant to 4-HPR treatment (Figure 1C).

4-HPR induces oxidative stress in 4-HPR-sensitive cells. The proapoptotic properties of 4-HPR have been shown to be related to its ability to induce ROS overproduction in the mitochondrial respiratory chain, which in turn triggers the intrinsic apoptotic pathway in CCRF-CEM and HeLa cells (8, 9). In the present work, the generation of ROS in different cell lines was measured after 4-HPR treatment. As expected, 4-HPR induced substantial oxidative stress in both the CCRF-CEM and Jurkat leukemia cells (Figure 2). In both these lines, ROS overproduction was dose-dependent, but the enhancement of ROS in CCRF-CEM cells at 10 μM 4-HPR was 4.0-fold, whereas it was 2.5-fold in Jurkat cells. A375 melanoma cells did not generate ROS in response to the retinamide.

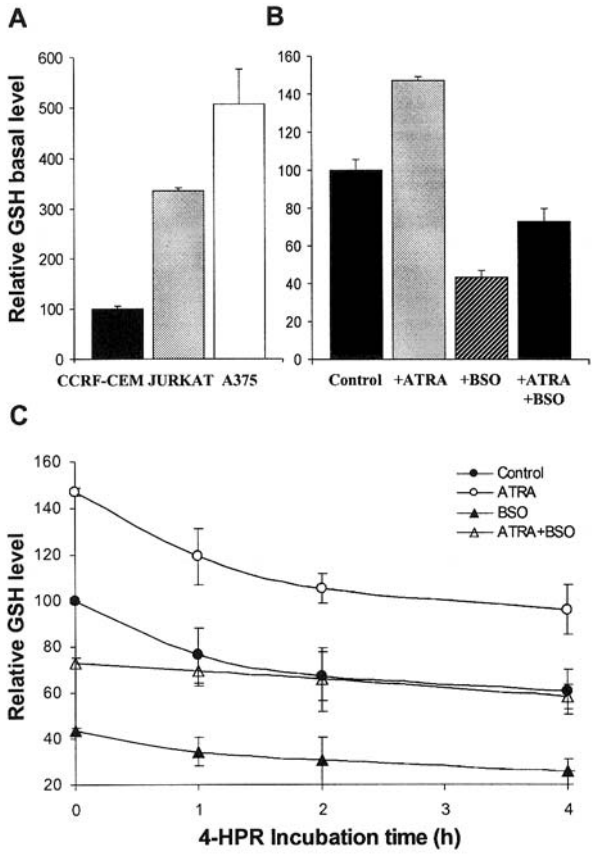

Figure 3. *Flow cytometry analysis of intracellular GSH content. (A) Basal levels of GSH in three different cell lines. (B) Basal levels of GSH in CCRF-CEM cells incubated with 1 nM ATRA for 48 h and/or 50 μg/ml BSO overnight. (C) Kinetic study of GSH content in CCRF-CEM cells incubated with ATRA (1 nM, 48 h) and/or BSO (50 μg/ml, overnight) following 6 μM 4-HPR treatment at indicated times. Relative values with respect to non-treated CCRF-CEM cells (100%) are shown. Values represent the mean ± SD of three independent experiments.*

Intracellular GSH levels are related to tumor cell sensitivity to 4-HPR. To investigate the possible role of intracellular GSH in the resistance of tumor cells to 4-HPR, the relative GSH contents in CCRF-CEM, Jurkat and A375 cells were compared. A375 cells showed 5-fold more GSH content than CCRF-CEM cells. GSH levels in Jurkat cells showed intermediate values (Figure 3A). These results suggest that the sensitivity of tumor cells to 4-HPR treatment is inversely related to the basal levels of GSH.

We also tested the consequences of modulating GSH levels in CCRF-CEM. To this end, we used BSO, which was found to considerably reduce GSH basal levels, and ATRA, which increased the intracellular level of GSH by almost 50%. Moreover ATRA was able to prevent GSH depletion produced by BSO (Figure 3B). As expected, 4-HPR-induced oxidative stress led to a significant fall in GSH content over

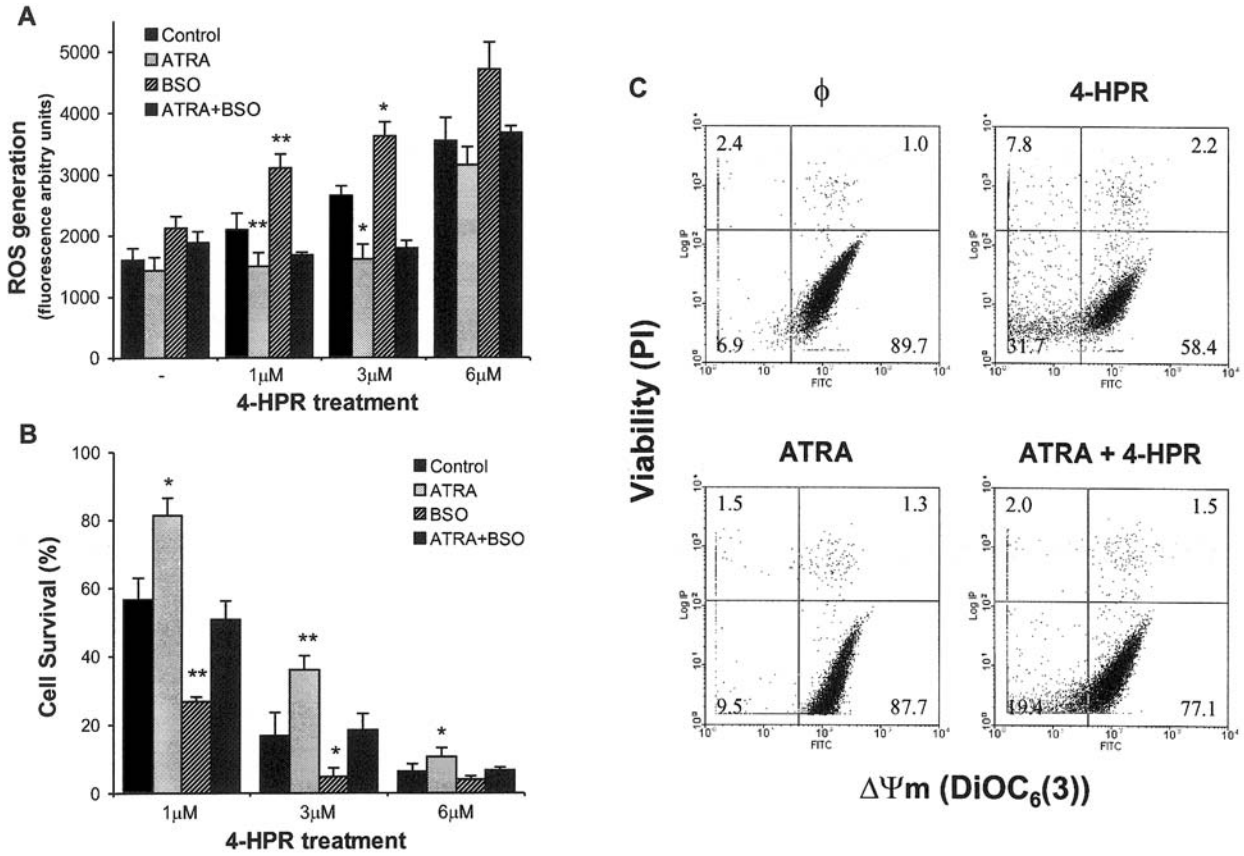

Figure 4. *Effect of modulation of GSH levels by ATRA or BSO on 4-HPR toxicity. CCRF-CEM cells were incubated with 1 nM ATRA for 48 h and/or 50 μg/ml BSO overnight and were then treated with increasing doses of 4-HPR. (A) Cell survival was determined by colorimetric assay after 24 h of 4-HPR treatment. Relative percentages with respect to non-4-HPR-treated cells are shown. (B) ROS generation was measured using DCFH-DA after 1 h of 4-HPR treatment. The means ± SD of three experiments are shown. Differences between ATRA- or BSO-treated cells and those only treated with 4-HPR were considered statistically significant when *p<0.05 and **p<0.01. (C) The reduction in ΔΨm was measured by flow cytometry after 8 h of 3 μM 4-HPR treatment. Representative dot plots of three experiments were carried out; numbers in quadrants indicate the percentage of cells.*

time (Figure 3C). However it was dramatically reduced in cells previously treated with BSO and to a lesser degree in the ATRA-treated ones (Figure 3C). In ATRA-treated cells, GSH levels always remained above basal /control ones even after 4 h of 4-HPR incubation.

Modulation of 4-HPR cytotoxicity by BSO and ATRA. CCRF-CEM cells were pre-treated with 1 nM ATRA for 48 h and then exposed to different concentrations of 4-HPR. The ability of 4-HPR to generate ROS was reduced in ATRA-treated cells (Figure 4A). Moreover, the capacity of 4-HPR to stimulate an apoptotic response (Figure 4B), mainly at 1 μM and 3 μM 4-HPR, was also reduced in ATRA-treated cells. Furthermore, the loss of mitochondrial membrane potential caused by 4-HPR was found to be reduced in ATRA-treated cells (Figure 4C). In contrast, GSH depletion caused by BSO was found to significantly enhance 4-HPR cytotoxicity. BSO increased 4-HPR-induced oxidative stress (Figure 4A) and cell death (Figure 4B) at all concentrations used. The effects of BSO on cell death were attenuated in the presence of ATRA, demonstrating the opposing effects of these agents. These results demonstrate that reducing GSH levels leads to the enhancement of the tumor cell response to 4-HPR.

Increased tissue transglutaminase expression in ATRA-treated cells. Several studies have recognized tTG as an important enzyme in the inhibition of cell growth and apoptosis (17, 18), although it has also been implicated in cell resistance to drugs (19, 20). It is known that ATRA induces cell growth inhibition and increases tTG activity in different cell types (21-23), because tTG is an ATRA target gene. To determine if this is also the case in CCRF-CEM cells, tTG expression levels were measured by Western blot after ATRA treatments (Figure 5). ATRA (1 nM) was found to increase tTG expression in CCRF-CEM cells. The increment of tTG

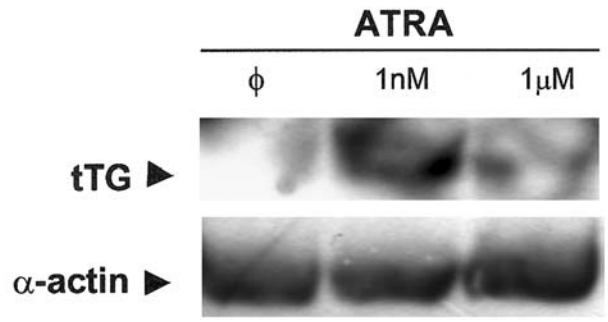

Figure 5. *ATRA treatment up-regulates tTG protein expression. CCRF-CEM cells were treated with 1 nM or 1 μM ATRA for 48 h and Western blot analysis of cell extracts was performed using a monoclonal anti-tTG antibody and an anti-actin antibody.*

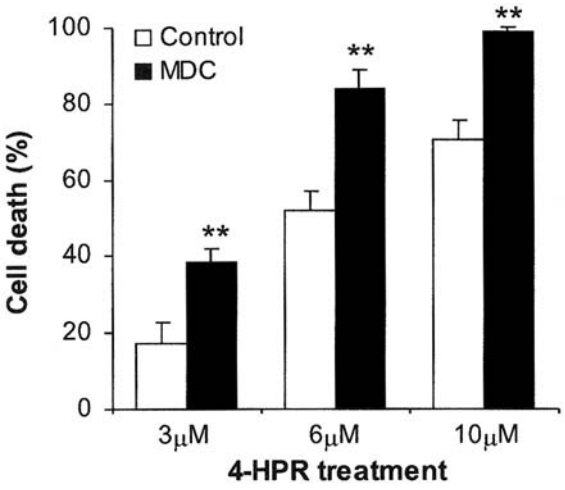

Figure 6. *Monodansylcadaverine (MDC) increased 4-HPR-induced apoptosis in Jurkat cells. Cells were pretreated with or without 70 μM MDC overnight. Subsequently, they were incubated for 96 h with increasing doses of 4-HPR. Values represent the mean ± SD of three independent experiments, each performed in quadruplicate. Differences in MDC-treated cells were statistically significant with respect to those treated only with 4-HPR (*p<0.05; **p<0.01).*

expression, as well as cell resistance to 4-HPR (data not shown), were less after treating cells with 1 μM than 1 nM. No detectable tTG expression was observed in control cells. These observations suggest that tTG expression may be an important feature of resistant cells.

Depletion of intracellular tTG levels by MDC increases ATRA and 4-HPR toxicity. In order to evaluate the consequence of tTG inhibition, CCRF-CEM cells were treated with the tTG inhibitor monodansylcadaverine (MDC). Simultaneous MDC and ATRA treatments results in generalized cell death (data not shown). Thus, ATRA is a potent cell death inducer in the presence of MDC. On the other hand, the inhibition of tTG expression by MDC renders Jurkat cells much more sensitive to 4-HPR. As shown in Figure 6, retinoid-induced apoptosis increased more than two-fold in MDC-treated Jurkat cells.

Discussion

The sensitivity and resistance of tumor cells to cytotoxic drugs depend on the activation of various routes to apoptosis, which in turn involve different signaling pathways. 4-HPR appears to exert its pro-apoptotic effects principally by increasing the levels of ROS (4, 5, 8, 9, 24). In this work, we have established a relationship among the response of different cell lines to 4-HPR, the oxidative stress produced by the treatments, and the endogenous antioxidative status of these cells. 4-HPR induces distinct effects in the studied cell lines. Thus, 4-HPR induced a quick dose- and time-dependent apoptotic activation in CCRF-CEM leukemia cells (Figure 1A), while in Jurkat leukemia cells the effect was smaller (Figure 1B). In addition, 4-HPR did not modify the survival of the A375 solid melanoma cell line (Figure 1C), although in cervical carcinoma and prostate cell lines it has been shown to produce significant effects (1, 2, 9). According to our results, 4-HPR induces oxidative stress only in sensitive cells (Figures 1 and 2), but not in the 4-HPR-resistant melanoma. Melanoma cells were found to express higher levels of GSH in comparison to leukemia cells. Moreover, in sensitive leukemia cells, a clear relationship was found to exist between the basal level of GSH and sensitivity / resistance to the retinoid (Figure 3A), indicating that the response to 4-HPR could be conditioned by the status of the endogenous antioxidant system. Tumor cells exhibiting increased GSH synthesis have been reported to be resistant to oxidative stress (12-15). GSH also plays an important role in the maintenance of mitochondrial function under stressful conditions of mitochondrial ROS-overproduction (11, 25, 26), as occurs in the 4-HPR-treated leukemia cells (8, 24).

Modulation of cellular GSH levels has been shown to lead to an alteration in the response of cells to some cytotoxic drugs (27, 28). GSH depletion caused by BSO led to increased ROS levels in CCRF-CEM cells (Figure 3B), as it does in other systems (11). Subsequently, apoptotic cell death caused by 4-HPR (Figure 4A and B) as well as other cytotoxic agents (29-31) is also enhanced under these conditions. In clinical trials, BSO has been demonstrated to enhance the cytotoxicity of some anti-neoplastic drugs such as melphalan (32). Our results would indicate that BSO should be considered as a candidate potentiating agent to combine with 4-HPR in clinical chemotherapy, in particular of leukemia.

We have also demonstrated that, in leukemia cells, basal GSH levels are increased after ATRA exposure (Figure 3B), which results in lower levels of 4-HPR-generated oxidative stress, reduced apoptosis-associated mitochondrial alterations (loss of $\Delta\Psi m$) and enhanced cell survival (Figure 4).

Together, these results suggest that ATRA can protect leukemia cells against the effect of 4-HPR by enhancing their antioxidative capacity. However, it has been reported that ATRA can induce both oxidant and antioxidant properties in AML cells (33). Moreover, the association of ATRA and 4-HPR has been reported to lead to increased 4-HPR antitumor activity in ovarian tumors (34). In contrast, our results demonstrate that ATRA reduces the cytotoxicity of 4-HPR in leukemia cells. Thus, the modulatory effect of RAs on intracellular antioxidants appears to be cell type-specific, and the associated mechanism of action remains to be elucidated. This cell type specificity should be taken into account when designing new clinical chemotherapy approaches using combinations of retinoids.

The protective effect of ATRA requires pretreating cells for a minimum of 48 h, suggesting that this effect is not as simple as the outcome of competing with 4-HPR for a common receptor (23, 35), but rather that both retinoids may activate distinct biological pathways. tTG may be a component in one of these pathways since ATRA treatments increased tTG expression (Figure 5). Increased tTG activity is commonly associated with the induction of programmed cell death (17, 18). In keeping with our results, other authors have demonstrated that increased tTG expression, induced by ATRA, protected HL60 cells and mouse fibroblasts from 4-HPR cytotoxicity (36, 37). It has recently been reported that enhanced tTG activity did not lead to increased cell death in a neuronal cell line (38) and that primary thymocytes isolated from tTG knockout mice seem to be more sensitive to cell death (39). In this sense, it has been proposed that the GTP binding activity of tTG may be sufficient to protect cells from apoptosis under certain conditions. Therefore, tTG might have two different functions with opposite effects: life and death (17). The concept of multifunctional proteins controlling apoptosis *versus* survival is neither unique nor novel, and highlights the complexity of these regulations.

Finally, increased levels of tTG in CCRF-CEM cells appear not only to be associated with the protective effect afforded by ATRA from 4-HPR-induced apoptosis, but its inhibition converts ATRA into a cell death signal, as has previously been reported (36). In addition, our results show that the inhibition of tTG activity in Jurkat cells can lead to an enhancement of 4-HPR-induced apoptotic cell death (Figure 6). Thus, it is conceivable that, in leukemia cells, tTG may function to ensure cell survival under certain conditions such as cell stress.

In summary, our results indicate that GSH levels may serve as a predictive factor of tumor sensitivity to 4-HPR-mediated apoptosis, and that the simultaneous use of both ATRA and 4-HPR may dramatically reduce the effectiveness of leukemia chemotherapy and should therefore be avoided in treatments of this type of cancer. In contrast, the efficacy of 4-HPR-based leukemia therapies may be enhanced by co-treatment with BSO.

Acknowledgements

We would like to thank Dr. Ignacio J. Ansotegui, from Santiago Apostle Hospital, Vitoria-Gasteiz, Spain, for his assistance with flow cytometry and data analysis of assays of surface antigens. This work was supported by grants from the University of the Basque Country (UPV 77.125-E-15360/2003 and UPV 75.327-14466/2002) and the Instituto de Salud Carlos III (FIS CO3/10). M. Celia Morales is supported by a fellowship from the Jesus de Gangoiti Barrera foundation and Naiara Rementeria received a fellowship from the University of the Basque Country UPV/EHU.

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Received December 8, 2004
Accepted March 22, 2005