Retinoic Acid Down-Regulates Aldehyde Dehydrogenase and Increases Cytotoxicity of 4-Hydroperoxycyclophosphamide and Acetaldehyde

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ABSTRACT

Multiple prior studies have identified aldehyde dehydrogenases (ALDH) that are capable of oxidizing retinal to retinoic acid. In this study, we test the hypothesis that the accumulation of intracellular retinoic acid may lead to the suppression of ALDH expression and thus increase cytotoxicity to 4-hydroperoxycyclophosphamide (4-HC) in vitro. Mainly A549, but also other lung cancer cell lines, were used in our experiments, with the former having high levels of two ALDH isozymes expressed. Dose-response and time-course experiments were performed by incubating the cells with all-trans retinoic acid (ATRA) as well as other commercially available retinoids. The results show that incubation of A549 cells with any of the retinoids at pharmacologic doses for 48 h results in a significant decrease in ALDH-1A1 and ALDH-3A1 enzyme activity and protein levels but not the corresponding mRNAs. Such a decrease in ALDH activity was seen in all cell lines tested and results in a significant increase in toxicity of 4-HC and acetaldehyde, both of which are substrates for the enzymes. Prior incubation with ATRA also results in increased cytotoxicity, although to a lesser degree, of phenylketophosphamide and melphalan, neither of which is a substrate for ALDHs. These results suggest a post-translational mechanism through which retinoids decrease both ALDH expression, which results in increased cytotoxicity of 4-HC and acetaldehyde, although other previously described effects of these retinoids may contribute to the slight increase in cytotoxicity seen with other chemotherapy agents. These results may have clinical implications in regard to the use of retinoids in lung cancer prevention and treatment.

Aldehyde dehydrogenases (ALDH) are a group of enzymes that catalyze the conversion of a broad range of aldehydes to the corresponding acid via a NAD⁺-dependent irreversible reaction. Two of these enzymes, cytosolic ALDH-1A1 and ALDH-3A1, have been found to be responsible for drug resistance in various tumor types against the antineoplastic drugs collectively known as oxazaphosphorines, which include cyclophosphamide and its active metabolites (Hilton, 1984; Manthey et al., 1990; Sreerama and Sladek, 1993; von Eitzen et al., 1994; Yoshida et al., 1998). Cyclophosphamide is a prodrug that requires cytochrome P450 hydroxylation for activation. Prior to the release of the active alkylating metabolite phosphoramidate mustard, cyclophosphamide passes through an aldehyde intermediate, aldophosphamide. ALDH oxidizes aldophosphamide to the inactive metabolite carboxyphosphamide (Manthey et al., 1990). Several inhibitors of ALDH activity have been used to demonstrate the reversal of this drug resistance mechanism. We and others (Bunting et al., 1994; Bunting and Townsend, 1996; Magni et al., 1996; Moreb et al., 1996, 1998) have successfully shown that overexpression of ALDH-1A1 or ALDH-3A1 in cell lines and normal hematopoietic progenitors results in a significant increase in the resistance to the active metabolites of cyclophosphamide in vitro. Furthermore, we have shown that down-regulation of ALDH-1A1 (Moreb et al., 2000) and ALDH-3A1 (J. S. Moreb and M. Schweder, unpublished data) using antisense RNA results in increased sensitivity of tumor cells to 4-hydroperoxycyclophosphamide (4-HC), an active derivative of cyclophosphamide.

Multiple prior studies have identified several ALDH that are capable of oxidizing retinal to retinoic acid (RA), a modulator of gene expression and cell differentiation that maintains numerous tissues (Gudas et al., 1994; Means and Gu-
das, 1995; Chytil, 1996). Human, mouse, and rat ALDH-1A1 have been reported to have high activity for retinal oxidation (Lee et al., 1991; Yoshiida et al., 1992; Bhat et al., 1995; Hsu et al., 1999). Thus, in this study, we test the hypothesis that the accumulation of intracellular retinoic acid may lead to the suppression of ALDH expression and thus increase 4-HC cytotoxicity in vitro. Our results suggest that retinoids indeed suppress levels of ALDH-1A1 and ALDH-3A1 protein and enzyme activity in physiologic and pharmacologic doses. The effects of retinoic acid on ALDH-1A1 and ALDH-3A1 provide new insight into the post-translational regulation of the enzymes.

Materials and Methods

Cell Lines. The A549 nonsmall cell lung cancer (NSCLC) cell line was obtained from the American Type Culture Collection (Rockville, MD). These cells were grown in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS) and used when in the exponential log phase of their proliferation. Other lung cancer cell lines used, including SW210.5 (small cell lung cancer) and ADLC-5M2 (NSCLC), were obtained from Dr. Gerald Bepler (H. Lee Moffitt Cancer Center, Tampa, FL) (Bepler et al., 1988, 1989).

Materials. All retinoids, all-trans RA (ATRA), and 9-cis and 13-cis retinoic acids were purchased from Sigma-Aldrich (St. Louis, MO). NAD, propionaldehyde, acetaldehyde, and melphanal were also purchased from Sigma-Aldrich. All retinoids were dissolved in ethanol. Melphanal was dissolved in small amounts of ethanol and acetic acid and then diluted in culture medium to the appropriate concentration.

4-HC and phenylethylketophosphamide (PKP) were supplied generously by Dr. Michael Colvin (Duke University, Durham, NC). 4-HC was dissolved in culture medium to obtain a 1-μg/ml solution. PKP was dissolved in dimethyl sulfoxide and then diluted in culture medium to obtain a 1-μg/ml final solution.

Chicken anti-human ALDH-1A1 and ALDH-3A1 polyclonal antibodies were generously provided by Dr. L. Sreerama (St. Cloud University, Minneapolis, MN) and Dr. N.E. Sladek (University of Minnesota, Minneapolis, MN). The specificity of these antibodies has been documented by Dr. Sladek’s groups (Sreerama and Sladek, 1993; Sladek et al., 2002).

RA Incubations. ATRA was the main retinoic acid used throughout the experiments. The other retinoic acids were used for comparison with ATRA. A dose-response effect on ALDH enzyme activity was performed using A549 cells after a 72-h incubation with a range of ATRA concentrations of 10⁻¹⁰ to 10⁻⁷ M.

A time-course experiment was also performed using A549 cells and a fixed ATRA concentration of 1 μM. ALDH enzyme activity was measured after a 12-h ATRA incubation and every 24 h up to 8 days. The starting cell concentration for A549 cells was either 0.5 × 10⁶ (up to 72-h incubations) or 0.1 × 10⁵ cells (for longer incubations) in 15 ml of RPMI 1640 in a 75-cm² flask. Cell count and viability were determined before any further use of the cells in the different assays. Viability was determined by the trypan blue exclusion criteria. Since ATRA is dissolved in 100% ethanol, control cultures received the same amount of ethanol as the ATRA-treated cultures in all the experiments.

Measurement of ALDH Activity. Measurement of the human ALDH activity was performed as described previously (Moreb et al., 2000). Briefly, cells were lysed in 0.5 to 1 ml of buffer containing 50 mM Tris (pH 8), 25 mM EDTA, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 0.1% sarcosyl. After centrifugation at 10,000 rpm for 10 min at 4°C, the supernatant was collected and used to determine ALDH activity. The protein concentration was determined using the BioRad protein assay kit with a bovine serum albumin standard (BioRad, Hercules, CA).

Aliquots of 600 μl incubated at 37°C using the Beckman DLC 64 cuvette spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) with the addition of protein extract, 5 μM NAD⁺, and 5 μM propionaldehyde (as substrate) were used to measure the change in absorbance at 340 nm over 5 min. A control reaction in which propionaldehyde was not added monitored the endogenous rate of NAD⁺ reduction.

Western Analysis. Western blot analysis was performed to demonstrate changes in the protein levels of ALDH-1A1 and ALDH-3A1. The same protein extract used for ALDH enzyme activity (described above) was used for the Western blot analysis. Equal amounts of protein from each experimental group were separated by size on a 10% denaturing SDS-polyacrylamide gel, electrotransferred onto nitrocellulose membranes, and labeled as described previously (Moreb et al., 2000). Chicken anti-human ALDH-1A1 and ALDH-3A1 primary antibodies were used at 1:200 dilution, and secondary antibody (horseradish peroxidase-labeled rabbit anti-chicken antibody; Sigma-Aldrich) was used at 1:6000 dilution. The chemiluminescence method (SuperSignal; Pierce, Rockford, IL) was used for the final visualization of the protein bands on X-ray film. All blots were labeled again for visualization of actin as a loading control using anti-actin antibody (Calbiochem, San Diego, CA).

Northern Analysis. Northern analysis was used to detect changes in ALDH-1A1 and ALDH-3A1 mRNAs after the incubation of cells with retinoic acid. Total RNA was extracted from A549 cells after different incubation times with ATRA using the Chomczynsky and Sacchi method (Chomczynsky and Sacchi, 1987). Total RNA (10 μg/lane) was separated on 1.2% agarose formaldehyde gel and blotted onto a nylon membrane (Micron Separations, Inc., Westboro, MA) by capillary transfer for 2 days. Detection of ALDH-1A1 and ALDH-3A1 was performed as described previously (Moreb et al., 2000), using random primer ³²P-dCTP-labeled ALDH-1A1 and ALDH-3A1 cDNAs.

In Vitro Drug Treatment Assay. After incubation with or without retinoic acid, cells were harvested. Following the determination of cell count and viability, A549, ADLC-5M2 (1 × 10⁶ cells/ml of fresh culture medium), or SW 210.5 cells (4 × 10⁵ cells/ml) were treated with 4-HC (40–80 μg/ml) for 30 min at 37°C, washed twice with culture medium, and then either plated for liquid colony assay (A549 cells only) or used for LDH cytotoxicity assay (see below) to determine the 4-HC cytotoxic effects.

To determine the specificity of retinoic acid effects on ALDH and sensitivity to 4-HC, we also treated A549 cells with drugs not metabolized by ALDH-1A1 or ALDH-3A1, such as PKP and melphanal. Again, cells with or without retinoic acid preincubation were treated with the corresponding drug for 30 min (PKP) or 60 min (melphanal) at 37°C, washed twice, and used for the LDH cytotoxicity assay.

Because acetaldehyde is a carcinogen metabolized by ALDH, we examined the effect of ATRA preincubation on the acute toxicity of a 24-h exposure of A549 cells to acetaldehyde. To determine the toxic dose of acetaldehyde, we first performed a dose-response experiment with acetaldehyde using the LDH cytotoxicity assay.

Cytotoxicity Assays. The Cytotoxicity Detection Kit was purchased from Roche Diagnostics (Mannheim, Germany) and used to measure the percentage of cytotoxicity by determining LDH activity in the supernatants of treated cells. We followed the manufacturer’s provided protocol. The optimal cell concentration per well and the optimal drug concentration were first determined by preliminary experiments to find the cell concentration that will give the maximal difference between the low and high controls.

A549 cells were also plated in a liquid colony assay to further measure the cytotoxic effects of 4-HC with or without retinoic acid preincubation as described previously (Moreb et al., 2000). After treatment, cells were resuspended in 4 ml of RPMI + 10% FBS and plated in four 35-mm Petri dishes for each experimental group. Colonies (>10 cells) adhering to the bottom of the plate were counted on day 4 using an inverted microscope. Untreated A549 cells were plated similarly at 200 cells/ml/dish. The toxicity of 4-HC was reflected by the amount of colonies killed and expressed as a percent-
Results

Regulation of ALDH Expression by Retinoic Acid. Because retinoic acid is the product of catalyzing aldehyde dehydrogenase, we incubated A549 cells with different ATRA concentrations and for different periods of time using 1 or 2 μM ATRA to test a possible negative feedback effect of high levels of retinoic acid on ALDH expression. The effect was measured by enzyme activity and Western and Northern analysis.

Figure 1 shows a dose-response effect on ALDH enzyme activity using ATRA 10^{-10} to 10^{-5} M. Because 10^{-6} M corresponds to the concentration achievable by pharmacologic doses of ATRA, we used that concentration in subsequent experiments. A time-course experiment revealed that ALDH enzyme activity decreases as early as 12 h after the beginning of incubation with 1 μM ATRA and continues as long as 8 days. The persistent decrease in ALDH activity depends on the addition of fresh ATRA every 48 h; otherwise, a rebound of ALDH activity is seen about 72 to 96 h after the initial addition of a single ATRA dose to the cell culture (data not shown). Also, in one experiment, we screened 13-cis and 9-cis retinoic acids for the longer incubation up to 8 days. Similar effects to that of ATRA on ALDH activity were seen by these two retinoic acids. Because of that, subsequent experiments were done with ATRA only. Table 1 and Fig. 2 show a significant decrease in ALDH activity and protein, respectively. Both ALDH-1A1 and ALDH-3A1 proteins are similarly affected on different days of the incubation (Fig. 2). Furthermore, the removal of ATRA after only a 3-h incubation will still result in a significant decrease in ALDH activity (20 ± 3%) at 48 h of continued culture of A549 cells, indicating that the ATRA effect is mediated through retinoic acid receptors and its signal transduction pathways.

Northern analysis of RNA obtained from A549 cells after a 72-h incubation with 1 μM ATRA showed no change in ALDH-1A1 or ALDH-3A1 mRNA levels (data not shown). These results suggest that the ATRA effect on ALDH levels is through post-translational regulatory mechanisms. During ATRA incubation, cell viability and count were determined at different times. A minimal effect on viability was noticed over an 8-day incubation (Fig. 3); however, a significant decrease in proliferation was noticed after a 5-day incubation with ATRA, as reflected by low cell counts compared with untreated cells (Fig. 4).

The use of other commercially available retinoic acids such as 13-cis and 9-cis retinoic acids resulted in similar effects to that of ATRA on ALDH enzyme activity in A549 cells (Table 1). Only one screening experiment with 13-cis retinoic acid was performed over 3, 5, and 8 days, and the results in Table 1 show similar but less robust effect on ALDH enzyme activity to that seen with ATRA. Subsequently, and in view of similar results obtained from 72-h incubations with the three different retinoic acids, we focused on performing studies using ATRA only.

To investigate whether the ATRA-induced decrease in ALDH activity is limited to the NSCLC A549 cell line, two other cell lines, NSCLC cell line ADLC-5M2 and small cell lung cancer cell line SW 210.5, were similarly incubated with ATRA for 72 h, and again, significant inhibition (P < 0.05) of ALDH activity was seen (Fig. 5), although the basal activity of ALDH in these cell lines was much lower than that in A549 cells.

Retinoic Acid Preincubation Results in Increased Sensitivity to 4-HC. Preincubation of A549 cells with 1 μM of ATRA or 13-cis or 9-cis retinoic acids for 3 days before treatment with 40 to 80 μg/ml 4-HC (Fig. 6, A) results in a significant increase in 4-HC toxicity (measured by LDH cytotoxicity assay) when compared with control cells. The percentage of release of LDH from the cells in the different groups before any 4-HC treatment was not different among the four experimental groups, indicating no significant toxicity caused by the retinoids themselves. Figure 6B shows a similar increase in 4-HC toxicity against A549 cells, as measured by liquid colony assay. To verify whether such an effect on 4-HC sensitivity correlates specifically to the decrease in ALDH enzyme activity, we treated A549 cells with a different ATRA concentration for 72 h and then with 80 μg/ml 4-HC. As expected, the results show that the increase in 4-HC cytotoxicity (as reflected by the relative amount of LDH release) corresponds to the degree of suppression of ALDH enzyme activity by the different ATRA dose level (Fig. 7, A and B). Furthermore, longer incubation (5 and 8 days) with ATRA or 13-cis retinoic acid does not generally result in more pronounced 4-HC toxicity (Table 2); rather, a plateau effect is seen. The LDH release at 0 μg/ml 4-HC was minimal and not significantly different between the cells in the three treatment groups in Table 2 (similar to what is shown in Fig. 6A), even after 8 days of incubation.

On the other hand, we used other cytotoxic drugs that are not metabolized by aldehyde dehydrogenase, such as PKP and melphalan, to further investigate the specificity of the retinoic acid effect. Table 3 shows that a 72-h ATRA preincubation results in increased cytotoxicity of PKP and melphalan but to a lesser degree than that seen for 4-HC.

The other two cell lines, ADLC-5M2 and SW210.5, were similarly treated with ATRA and 4-HC. Toxicity was measured by LDH cytotoxicity assay. The results showed increased sensitivity to 80 μg/ml 4-HC toxicity by 23 to 50% over that seen with the control cells with no prior ATRA incubation.
viability measured in at least three similar experiments. The results of days 5 and 8 for 13-cis retinoic acid reflect the mean ± S.D. of four readings from one experiment. In each raw experiment, the top value is calculated based on activity measured as nanomoles per milligram of protein per minute, whereas the bottom value is calculated based on activity measured as nanomoles per 10^7 cells/min; n, number of experiments.

### TABLE 1

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**Fig. 2.** Western blot analysis showing a decrease in ALDH-1A1 and ALDH-3A1 proteins at different time points of incubation of A549 cells with 10^-M ATRA. ATRA was added every 48 h for continued effect. Control (C) A549 cells were treated with an equivalent volume of ethanol because it was used to dissolve the ATRA.

**Fig. 3.** Effect of ATRA incubation on A549 cell viability. 10^5 cells/experimental group were incubated with (dark bars) or without (light bars) 10^-M ATRA for 3, 5, and 8 days. Cell viability was determined by trypan blue exclusion criteria. Each bar represents mean ± S.D. of viability measured in at least three similar experiments.

**Fig. 4.** Effect of ATRA on A549 cell proliferation as reflected by the -fold increase of total viable cells over time. 10^5 cells/group were incubated with (dark bars) or without (light bars) 10^-M ATRA for 3, 5, and 8 days. Cell count and viability was determined at each time point, and the -fold increase in viable cell number was assessed in comparison with the starting cell number. Each bar represents mean ± S.D. of the -fold increase in cell number from at least three different experiments. The differences between the ATRA and the control groups were significant on days 5 and 8 of incubation (P < 0.025).

**Fig. 5.** Effect of ATRA incubation on ALDH enzyme activity in two different lung cancer cell lines.

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**Increased Acetaldehyde Cytotoxicity after ATRA Incubation.** Because of the use of carotinoid derivatives in cancer prevention and because acetaldehyde is a known carcinogen metabolized by ALDH, we examined the effects of the ATRA-induced decrease in ALDH enzyme activity on the cytotoxicity of acetaldehyde. Figure 8A shows partial results of the dose-dependent toxicity against A549 cells after a 24-h exposure to acetaldehyde. Doses of 200, 400, and 800 μM had zero toxicity. Figure 8B shows significant (P < 0.025) increase in the cytotoxic effects of acetaldehyde (20 mM for 24 h) on A549 cells after a 72-h incubation with any of the three retinoic acids used here.

Although the concentrations of acetaldehyde used here seem very high, it is important to know that A549 cells are relatively resistant and that treatment with 5 to 20 mM acetaldehyde for 24 h is 100% lethal to an immortalized normal epithelial cell line, IB-3 (Flotte et al., 1992), that has minimal ALDH activity (data not shown).

**Discussion**

ALDH-1A1 has been shown to catalyze retinaldehyde into retinoic acid in several tissues (Lee et al., 1991; Yoshida et al., 1992; Gudas et al., 1994; Bhat et al., 1995; Means and Gudas, 1995; Chytil, 1996; Hsu et al., 1999). In this report, we show that incubation of lung cancer cell lines with any of three different retinoic acids results in a significant decrease in ALDH-1A1 and ALDH-3A1 protein and enzyme activity. The decrease seems to take place at the post-translational level since retinoic acid did not affect the mRNA levels for both enzymes. Although a significant decrease in ALDH activity is seen with very low retinoic acid concentrations of 10^-10 M, further dose escalation did not show a significant drop in activity until toxic ATRA concentrations of 10^-5 M. These observations are significant since a 10^-5 M concentration of ATRA is clinically unattainable and unfeasible. Furthermore, it seems that the decrease in ALDH activity is significant with a wide range of ATRA concentrations. Using a therapeutic concentration of ATRA (1–2 × 10^-6 M) over 8 days shows that the decrease in ALDH enzyme activity occurs as early as 12 h after the initiation of retinoic acid
exposure and continues to drop as late as 8 days, but the best and most consistent effect on 4-HC cell sensitivity seems to be between days 3 to 5 of retinoic acid incubation. Such a plateau effect on the 4-HC sensitivity could be related to other compensatory mechanisms or an artifact of the culture system.

The viability data (Fig. 3) show minimal direct toxicity by ATRA itself but more pronounced inhibition of proliferation (Fig. 4). This observation suggests a possible cell cycle inhibition during prolonged incubation with ATRA. These ATRA effects that may vary from one cell type to another have been described and published previously (Sun et al., 1999; Lutz et al., 2001; Sun and Lotan, 2002; Hayashi et al., 2003). It is possible that the above-mentioned plateau effect on 4-HC toxicity beyond 5 days of continuous incubation with ATRA is associated to such proliferation inhibition.

We believe that this is the first report to describe a mechanism by which retinoic acid may enhance the efficacy of a chemotherapeutic agent such as 4-HC. However, few previous studies reported such effects of retinoic acid on other cytotoxic drugs, including cisplatin, etoposide, paclitaxel, and gemcitabine (Formelli and Cleris, 1993; Shalinsky et al., 1996; Grunt et al., 1998; Kalemkerian and Ou, 1999; Pettersson et al., 2001). The basis for those studies was the known antitumor effects of retinoic acids. Thus, it was important to study the specificity of the ATRA-induced enhancement of the cytotoxic effects of 4-HC against A549 cells. Our studies show that ATRA preincubation results in increased sensitivity to melphalan and PKP, both of which are not metabolized by ALDH-1 or ALDH-3, but to a much lesser degree than that seen with 4-HC. Thus, ATRA or other retinoic acids seem to have an additional yet unknown effect other than that on ALDH. These results are not surprising because retinoids have been known for years as potent agents that control cellular proliferation, differentiation, and apoptosis through a wide array of signal transduction pathways.

Acetaldehyde is a known carcinogenic aldehyde that could be found in cigarette or car exhaust smoke as well as a product of ethanol metabolism. Acetaldehyde plays an important role in the pathogenesis of tissue injury that results from alcohol and cigarette consumption (Feron et al., 1991; Koivisto and Salaspuro, 1997). Since acetaldehyde is a substrate for aldehyde dehydrogenase, the presence of high levels of ALDH could protect against the toxicity of acetaldehyde, and vice versa. Our studies show that the ATRA-induced reduction in ALDH-1A1 and ALDH-3A1 results in increased acetaldehyde toxicity. Similar interaction was re-
ported between β-carotene and acetaldehyde and was attributed to the common metabolic pathway, possibly via ALDH (Ni et al., 2001).

Our current results should open the way to new studies that further evaluate the regulatory mechanisms involved in the retinoic acid-induced reduction in ALDH-1A1/ALDH-3A1 levels. The fact that the ALDH-1A1/ALDH-3A1 mRNA’s were not reduced by ATRA indicates a possible post-translational mechanism that results in decreased protein levels and enzyme activity of these isoforms. It is possible that our initial hypothesis about a retinoic acid-mediated negative feedback might be true, but the possibility that ALDH inhibition may be induced indirectly through another retinoic acid-known biological effect seems more likely, and further investigation will be needed. Indeed, retinoic acid was reported to reduce p11 protein (Gladwin et al., 2000), cyclin D1 (Langenfeld et al., 1997), and retinoic acid receptors α and β by inducing degradation through the ubiquitin-proteasome pathway, a post-translational mechanism. Thus, it is possible that ALDH-1A1 and ALDH-3A1 are also regulated by proteolysis through the ubiquitin-proteasome system or other mechanisms and that the retinoic acid effect on their levels is mediated through increased protein degradation.

In summary, we have shown that different forms of retinoic acids reduce the ALDH-1A1 and ALDH-3A1 proteins and enzyme activity via a post-translational mechanism and increase the cytotoxicity of 4-HC and acetaldehyde in lung cancer cell lines. Based on these observations, it will be reasonable to pursue clinical trials combining ATRA and cyclophosphamide in the treatment of lung cancer and other malignancies. Furthermore, our observations could serve as the basis for studying and revealing the regulatory mechanisms involved in determining the cellular expression of these enzymes.

References